PLASMA MICROPARTICLES IN PATIENTS WITH GYNAECOLOGICAL MALIGNANCY

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DECLARATION

In accordance with the postgraduate degree regulations of the University of Edinburgh, I declare that this thesis has been written solely by myself, that the work described here is my own and that it has not been submitted for any other degree.

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SEPTEMBER 2010

ABSTRACT

Gynaecological malignancy is a common diagnosis that is highly amenable to effective treatment. Unfortunately the incidence of thrombosis, together with its morbidity and mortality, remains significant in this patient group. Patients with thrombosis have also been shown to have a worse prognosis. Thromboprophylaxis can reduce the incidence of thrombosis and may potentially also improve outcomes. Primary thromboprophylaxis could be indicated in patients considered to be at high risk of thrombosis. However empirical thromboprophylaxis is not without its risks as cancer patients also have a significant risk of bleeding. Primary thromboprophylaxis cannot at present be recommended for all patients with gynaecological malignancy. We are currently unable to assess whether or when a thrombosis is likely to happen. The ability to identify patients at a high risk of thrombosis would allow a more tailored approach to primary thromboprophylaxis that could translate into improved outcomes.

Microparticles are postulated to be important participants in many pathophysiological processes, including haemostasis and thrombosis. There is also increasing evidence that microparticles influence cancer cell survival, invasiveness and metastasis. There are several publications in peer reviewed literature reporting increased numbers of circulating plasma microparticles in patients with malignancy and other prothrombotic conditions. It is hypothesised that microparticles may be directly involved in the pathogenesis of thrombosis and in cancer cell survival and progression, and hence may have a direct effect on overall prognosis and outcome. Measuring the number of circulating plasma microparticles could potentially help identify cancer patients at increased risk of thrombosis that may benefit from primary thromboprophylaxis, which in turn could potentially lead to improved overall outcomes.

I set out to quantify and compare the number of circulating plasma microparticles in patients with gynaecological malignancy and a control group of women with no

malignancy. I also wanted to establish whether measuring the number of plasma microparticles would identify patients at an increased risk of thrombosis.

Research on microparticles is currently hampered by the lack of a standardised method for the identification and quantification of microparticles, mainly due to their small size. There are several pre-analytical variables that may influence the number of microparticles identified. These include the isolation methods, the labelling antibodies and the assays used to quantify microparticles, amongst others. Use of different laboratory techniques by different investigators makes the meaningful comparison of results in the published literature difficult.

Flow cytometry is the technique most commonly used to quantify microparticles. I set up a laboratory assay based on light-scattering flow cytometry to identify and quantify microparticles. Platelet free plasma was prepared from citrated blood, monoclonal antibodies were used to label microparticles, and a flow based method used to quantify the absolute number of microparticles in the patient plasma by flow cytometry.

There is currently still debate as to whether microparticle assays need to be done on fresh plasma samples or whether they can be meaningfully done on frozen samples. In the first twenty patients I compared the results obtained from fresh plasma with the results from a separate aliquot of the same plasma sample that had been stored frozen at -80°C. The number of microparticles detected after a freeze/thaw cycle did not correlate with the results obtained from fresh plasma. In view of this I analysed all patient samples on fresh plasma within two hours of collection. All patient samples in my study have been handled in an identical manner to ensure that results from different patients are comparable.

I recruited 67 women with gynaecological malignancy and a control group of 42 women without malignancy. I measured the total number of circulating platelet-derived microparticles, leucocyte-derived microparticles, endothelial-cell derived microparticles, tissue factor positive microparticles and annexin V positive

microparticles. There was no statistically significant difference in the number of circulating microparticles between the patients with gynaecological malignancy and the women without malignancy. Five (7.5%) of the patients with gynaecological malignancy were diagnosed with a venous thrombotic event during the study follow up period (median of 11 months, range 5-17 months). The number of plasma microparticles in the latter patients was indistinguishable from that in gynaecological cancer patients without thrombosis and in the control group. However, because only a small number of patients (five) were diagnosed with a thrombosis, it has not been possible to ascertain whether such patients truly do not have raised levels of microparticles, or whether a larger study might demonstrate a difference.

In order to establish whether my study cohort is representative of gynae-oncology patients in the South East of Scotland Cancer Network (SCAN), I identified all patients diagnosed with a gynaecological malignancy in SCAN during 2009 (period of study recruitment). I compared the characteristics of this group of patients with my study cohort and found that my study cohort was highly representative of the overall population of patients with gynaecological malignancy in SCAN with a similar frequency of the different gynaecological malignancies and a similar incidence of thrombosis.

There are a number of limitations to this study. There is still no universally accepted definition of what constitutes a microparticle. The assay I have used, while standardised for this study, is not universally used by all investigators measuring microparticles. Although flow cytometry is the commonest technique used to measure microparticles, the accuracy of the assay is called into question given the small size of the microparticles being measured. Further this is a single centre study, and although all the patients recruited had been diagnosed with gynaecological malignancy, given the variable nature and prognosis of the different types of gynaecological cancers, the patients recruited are in fact a diverse group. Recruiting patients with a specific type of gynaecological cancer only would have led to a more uniform group of patients that are more readily comparable. The overall number of patients diagnosed with a venous thromboembolic event, while representative of the

overall population in SCAN diagnosed with gynaecological cancer, is too small to be able to draw any firm conclusions regarding the usefulness of measuring microparticles as a prothrombotic marker or otherwise, particularly given the different incidence of venous thrombotic events in the different types of gynaecological cancer.

As far as I can establish this is the first study looking at microparticles specifically in patients with gynaecological malignancy. Keeping the limitations of the study in mind, I did not find a difference in the number of plasma microparticles in patients with gynaecological malignancy compared to a control group of women with no cancer. Further, microparticles cannot at present be used to help identify patients with gynaecological malignancy at a higher risk of venous thrombotic events.

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APC	allophycocyanin
AV	annexin V
bFGF	basic fibroblast growth factor
BSO	bilateral salpingo-oophorectomy
ca	carcinoma
CIN	cervical intraepithelial neoplasia
CT	computerised tomography
CV	coefficient of variation
CVA	cerebrovascular accident
DVT	deep vein thrombosis
Dx	diagnosis
ECM	extracellular matrix
EGFR	estimated glomerular filtration rate
ELISA	enzyme linked immunoassay
EMP	endothelial cell derived microparticle
FBC	full blood count
FITC	fluorescein isothiocyanate
FSc	forward scatter
FX	factor X
GP	glycoprotein
Hb	haemoglobin
HUVECs	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule 1
IL-6	interleukin-6
IT	information technology
LETZ	loop excision of transformational zone
LMP	leucocyte derived microparticle
MDM	multidisciplinary meeting
MI	myocardial infarction
MMP	matrix metalloproteinase

MP	microparticle
No.	number
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PE	R-phycoerythrin
PFP	platelet free plasma
PPP	platelet poor plasma
PMP	platelet derived microparticle
PS	phosphatidylserine
Pt	patient
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RMP	red cell derived microparticle
RT	radiotherapy
Rx	treatment
SCAN	South East of Scotland Cancer Network
SSc	side scatter
TED	thromboembolic deterrent
TAH	total abdominal hysterectomy
TF	tissue factor
TNFα	tumour necrosis factor α
TMP	tumour cell derived microparticle
uPA	urokinase-type plasminogen activator
VAIN	vaginal intraepithelial neoplasia
VEGF	vascular endothelial growth factor
VIN	vulval intraepithelial neoplasia
VTE	venous thromboembolism
vWF	von Willebrand factor
wbc	white blood cells

STATISTICS

Using the Shapiro-Wilk test, the variables measured in this study (microparticle numbers, markers of haemostatic activation and haematological and renal parameters) were found not to be normally distributed. Values are therefore expressed as the median and the interquartile range (IQR). Comparisons between two independent groups of patients were carried out using the Mann Whitney *U* test, while comparisons between three or more independent groups were carried out using the Kruskal Wallis test. Multivariate analysis was also carried out to allow for age adjustment.

The correlation between different variables has been assessed using Spearman's nonparametric correlation coefficient (r). Paired samples were compared using the Wilcoxon Signed Ranks test. All reported probability values are two-tailed. Statistical significance was defined as a p value of < 0.05. Analyses were performed with the statistical package SPSS version 17.0 (SPSS Inc, Chicago, IL, USA).

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CHAPTER 1

INTRODUCTION

GYNAECOLOGICAL MALIGNANCY, THROMBOSIS AND MICROPARTICLES

1.1 GYNAECOLOGICAL MALIGNANCY

1.1.1 Introduction

Gynaecological malignancies consist of ovarian cancer (which includes fallopian tube cancer and primary peritoneal cancer), endometrial cancer, cervical cancer, vaginal cancer and vulval cancer. Together they are the third[1] commonest (after breast and colorectal cancer) diagnosis of cancer in women in the developed world (second commonest in the developing world), with the overall incidence worldwide being approximately 1 million women per annum[2]. Gynaecological cancers are highly amenable to effective treatment. The 10 year survival rate varies according to the specific type of gynaecological malignancy, being 75% for endometrial cancer, 62% for cervical cancer and 36% for ovarian cancer[3].

The management of individual patients with gynaecological cancer varies according to the specific cancer site (i.e. ovarian, cervix, vaginal, vulval, endometrial), its histological subtype (e.g. squamous carcinoma, mucinous carcinoma), the clinical stage and any co-morbidities the patient may have. Treatment normally includes any or all of surgery, chemotherapy and radiotherapy (external beam radiotherapy and/or brachytherapy)[4].

Unfortunately VTE (DVT and/or pulmonary embolism) remains a complication in this patient group, occurring either as a presenting feature or as a complication of treatment or disease progression[5-10]. The management of VTE events in cancer patients has improved significantly over recent years; however VTE remains the second commonest cause of death in this group of patients[5].

1.1.2 VTE in cancer patients

1.1.2.1 Incidence of VTE

Cancer patients have a 4-5 fold (in some studies up to 7-fold)[11-15] increased risk of VTE. Patients with cancer represent approximately 15-20% of all patients presenting with a VTE[13-15], and 20% of all cancer patients will experience a VTE at some point[16]. The highest incidence of VTE is reported in patients with malignancies of the ovary, pancreas and glioblastomas[5, 17, 18]. The risk of VTE is highest in the first year after the malignancy is diagnosed, reflecting both the presence of a malignancy and its treatment; in the first 3 months the risk is increased 53-fold, and then declines thereafter. By 2 years after the diagnosis of cancer the relative risk of VTE decreases considerably, but remains higher than that of the general population, and it is only after 15 years that the risk subsides[7, 11, 19]. This seems to be true both for patients with only a lower limb DVT and for patients with a pulmonary embolism with/without lower limb DVT. Most thrombotic events involve the venous system, although arterial thromboembolism has also been reported[20-22].

The risk of VTE is also linked to the extent of disease. The relative risk of VTE in patients with metastasis is increased 58-fold compared to patients with no malignancy, a much higher risk than for patients with cancer but no metastases (4-fold)[11]. The presence of metastases is associated with a hypercoagulable state as the haemostatic system plays an important role in the ability of solid tumours to metastasise (see section 1.1.2.3, page 6).

Unfortunately there is little in the way of prospective data assessing the incidence of VTE events in cancer patients through systematic screening; the estimates available are derived from autopsy series, epidemiological surveys, and prospective chemotherapy trials. The quoted incidence of VTE events in patients with gynaecological malignancy is very variable: 13.6-27% of ovarian cancer patients[23], 9.8-57% of patients with endometrial cancer[8, 24, 25] and 0-34% of patients with cervical cancer[9]. The rate of pulmonary embolism is reported to be 1-2.6%, and goes up to 6.8% in post-operative ovarian cancer patients[6].

In a significant proportion of patients with gynaecological malignancy clinically silent VTE is detectable prior to any treatment; please refer to Table 1.1[8, 26]. Such asymptomatic VTE present prior to surgery may progress and become symptomatic postoperatively, partly contributing to the high incidence of VTE in the postoperative period in patients with gynaecological cancer. Clinically asymptomatic DVT has been found in as many as 25% of patients with ovarian cancer and 9.9% of patients with endometrial cancer. However there are several studies that have documented an even higher incidence of postoperative VTE in patients with endometrial cancer[24, 27-29], so that the incidence of asymptomatic VTE prior to surgery is unlikely to fully account for the high incidence of VTE postoperatively in such patients[8]. In these patients VTE often occurs during treatment in the absence of prior asymptomatic VTE.

	Endometrial cancer	**Ovarian cancer**	**Cervical cancer**
Clinically silent DVT	9.9%	25%	5.2%
Clinically silent DVT with PE	4.7%	11.1%	1.2%

Table 1.1: Incidence of clinically silent venous thromboembolism (VTE) prior to treatment in patients with gynaecological malignancy[8]. Deep vein thrombosis (DVT), pulmonary embolism (PE).

1.1.2.2 Pathophysiology of VTE

Despite the strong association between malignancy and VTE, the pathophysiological basis of this relationship remains uncertain. Virchow's triad of endothelial damage, blood stasis, and hypercoagulable state leading to VTE remains valid today[30]. In patients with gynaecological malignancy thrombosis occurs as a result of the interaction of many different variables including a) patient specific factors, b) tumour related factors and c) extrinsic factors related to the treatment of the tumour (surgery, chemotherapy, radiotherapy).

The patient specific factors that contribute to the risk of thrombosis are many and variable, including older age, increased BMI, immobility, genetic prothrombotic defects (such as factor V Leiden and antithrombin deficiency[20]), sepsis, smoking, hypertension and diabetes.

The tumour related prothrombotic factors are also many and variable. They include the physical characteristics of the tumour such as the size and site of any pelvic masses that may be compressing (causing blood stasis) and damaging the endothelium of blood vessels, as well as the characteristics of the tumour cells and the tumour microenvironment. Tumour cells can up-regulate coagulation factors, down-regulate the fibrinolytic system, and express cytokines or regulatory proteins associated with clot formation[31]. Tumour cells can also secrete coagulation factors themselves (e.g. TF, thrombin) so that monocytes and endothelial cells in the tumour microenvironment can also promote thrombosis[20, 32-34]. The prothrombotic state of cancer patients is due to an imbalance between the procoagulant and anticoagulant systems of the body, damage to the endothelium of blood vessels, and activation of platelets[20].

Treatment of the malignancy also contributes to the prothrombotic state of cancer patients[7, 35-40]. Treatment of gynaecological cancer can include any or all of surgery (often complex abdominal and pelvic surgery), chemotherapy and radiotherapy. These treatment modalities lead to a prothrombotic state through the up-regulation of procoagulant proteins, down-regulation of anticoagulants

(antithrombin, protein C, protein S), suppression of the fibrinolytic system, increased platelet activation, enhanced adhesion of neutrophils and the release of cytokines and tumour procoagulants during tumour cell lysis[6, 41-46].

1.1.2.3 Thrombosis and cancer prognosis

VTE often occurs prior to the diagnosis of cancer, and patients with cancer who are diagnosed with a thrombotic event have a worse prognosis[19, 20, 47]. There is a vicious cycle in which hypercoagulability facilitates the aggressive behaviour of malignancy and vice versa. The clotting and fibrinolytic pathways influence tumour growth[48, 49]; coagulation factors have been found to have a profound effect on tumour cell behaviour both in *in vivo* and *in vitro* studies, enhancing tumour cell proliferation, invasion, angiogenesis, and metastasis[20]. The prothrombotic state found in patients with malignancy seems to affect the ability of tumour cells to survive, proliferate and disseminate[20, 48, 49].

Thrombotic episodes are associated with significant morbidity and mortality. Patients presenting acutely with pulmonary embolism have an overall all cause 30-day mortality of approximately 3%[13, 50-53]. The natural history of thrombosis is more aggressive in patients with underlying cancer. The mortality rate in such patients is higher than in patients with either cancer alone or thrombosis alone[6, 7, 12, 19, 20, 54]. Patients in whom cancer was diagnosed within a year of a thrombotic event are more likely to have advanced disease and a poor prognosis than patients with cancer but no thrombosis[19]. Survival is particularly poor when the diagnosis of cancer is concurrent with the thrombotic event; only 12% of patients diagnosed with cancer at the time of a DVT were alive at 1 year[7, 19], while the overall survival for patients in whom cancer was diagnosed more than one year after VTE was similar to that for matched (for type of cancer and age) patients without VTE. These findings indicate that VTE in a patient with cancer suggests the presence of advanced and biologically aggressive disease; it is unlikely that the increased mortality in patients with malignancy and VTE is purely a complication of VTE. A number of studies suggest that anticoagulant therapy may reduce the incidence of cancers, hinder their progression and decrease the mortality rate[19, 20, 55-60],

although not all studies have confirmed these findings[19, 61]. The exact mechanism by which heparin mediates antitumour or antimetastatic activity is unclear, however these findings raise the question of whether patients with VTE and cancer should receive more aggressive anticoagulation than other patients with VTE but no cancer. The relatively poor prognosis of cancer diagnosed soon after VTE also suggests that more aggressive anticoagulant therapy might be appropriate in such patients[19]. Further studies are needed to explore the interaction between the clotting system and cancer progression. In the future, treatment targeting activated clotting factors may be part of a viable strategy for treating cancer.

If cancer patients at a significantly increased risk of thrombosis could be prospectively identified the use of primary thromboprophylaxis in this subgroup of patients could reduce the risk associated with thrombotic events, and potentially improve the prognosis of the malignancy. Thromboprophylaxis with mechanical compression devices and/or heparin has been shown to significantly reduce the rate of VTE (both DVT and pulmonary embolism) in patients undergoing surgery for gynaecological cancer[6, 62-64], which in turn translates into a reduced mortality rate, so that thromboprophylaxis is indicated in all patients undergoing such surgery. However there is very little data on the use of primary thromboprophylaxis in the non-surgical setting. At present we have no way of predicting whether or when thrombosis will occur. Laboratory markers of coagulation activation are often elevated in patients with cancer[18] but are of little clinical use in assessing the individual risk for thrombosis. The use of thromboprophylaxis is not without its risks: the risk of major bleeding is 2-6 fold higher in patients with cancer[11, 12, 65-67]. If we were able to identify subgroups of patients at increased risk of thrombosis, then treatment with primary thromboprophylaxis could be targeted.

1.2 MICROPARTICLES

1.2.1 Definition and formation of MPs

In 1949 Chargaff[68] first recognised that platelet-free plasma (PFP) contains a precipitable factor that can accelerate thrombin generation. In 1967, Wolf noted the presence of cell-membrane derived material in PFP (obtained by ultra-centrifugation) capable of generating thrombin[68, 69] – electron microscopy confirmed that these small microvesicles originated from activated platelets; at the time Wolf described them as "platelet dust". In 1999 Combes described EMPs derived from HUVECs after TNFα stimulation[68, 70]. These MPs were detectable in both healthy individuals and patients with prothrombotic conditions, and they expressed the same antigens as their cell of origin. Since then there has been a lot of research carried out to further define MPs and to try to establish their properties and functions.

MPs are described as intact phospholipid vesicles, 0.1-1.0μm in diameter[71], released from the cell membranes of most cells (platelets, leucocytes, endothelial cell, tumour cells) by budding of parts of the outer cell membrane[18, 72]. They are a heterogeneous group, bearing cell-specific surface antigens of their cell of origin, and lacking a nucleus. They are distinct from apoptotic bodies and exosomes[73], the latter being formed intracellularly and released by fusion with the cell membrane, while apoptotic bodies are formed during the final stages of programmed cell death and are generally larger than MPs. The release of MPs is a physiological process, with MPs being detectable in all individuals. Their levels are reported to be higher in females and are affected by the menstrual cycle and the circadian rhythm[73-75].

Most investigators have reported that in healthy individuals PMPs make up the majority of MPs (70-90% of all circulating MPs), with LMPs, EMPs and RMPs making up the remainder[71, 76, 77]. More recently Shah found that in healthy individuals the major sources of MPs were endothelial cells and platelets, contributing to 43.8% and 38.5% of all MPs respectively[78, 79]. When one considers the total endothelial cell surface area (average adult ~350m^2), it would not be surprising if EMPs do predominate in the circulation[78, 80]. These differences

in the published literature may reflect variability in the underlying medical conditions; however the method of sample preparation and processing is also likely to be contributing to the variable results by different investigators. The profile of circulating MPs does seem to alter with disease states. In patients with malignancy TMPs can also be detected, and analysis of such TMPs could have a diagnostic and possibly therapeutic potential. Patients with malignancy and/or thrombosis are known to have evidence of endothelial dysfunction, vascular inflammation and a prothrombotic state, and as might be expected elevated levels of MPs have been reported in these conditions[18, 23, 81-83].

The number of circulating MPs depends on the balance between their rate of release from cells and their clearance from the circulation. Cell activation results in an increase in the rate of MP release. The method of MP clearance from the circulation is less clear. Platelets have a life span of approximately 10 days, much longer than that reported for PMPs of about 30 minutes in mice[73, 76] or less than 10 minutes in rabbits[73, 84]. Possible mechanisms postulated to lead to the clearance of MPs include: 1) by phospholipases[85], 2) phagocytosis after PS exposure, or 3) indirectly such as by opsonization by proteins such as protein S and complement[86].

The rate of MP release increases in response to several different stimuli including: 1) cell activation or apoptosis, 2) complement mediated cell lysis and cytokines, 3) oxidative injury and 4) other insults such as high shear stress[87, 88]. An increase in the intracellular concentration of calcium seems to be a necessary triggering event or common pathway for vesicle release. The current knowledge on MP formation is mainly derived from *in vitro* experiments, with the *in vivo* mechanisms involved in MP formation still mainly unknown.

In the steady state, the cellular membrane is asymmetrical with phosphatidylcholine and sphingomyelin found mainly in the outer layer, and PS and phosphatidyl-ethanolamine being present in the inner layer. The enzymes flippase, floppase and scramblase are responsible for maintaining this membrane asymmetry. During cell activation or apoptosis, the endoplasmic reticulum releases calcium. The increase in

intracellular calcium inactivates flippase and activates floppase and scramblase, leading to a loss of the phospholipid asymmetry of the cell membrane and to disruption between the phospholipids and the cytoskeleton. The calcium also activates calpain and gelsolin which cause disruption of the proteins anchored to the cytoskeleton, leading to membrane budding and MP shedding (microvesiculation)[73, 78, 89]; please refer to Figure 1.1, page 11. Such MPs are rich in phosphatidylethanolamine and PS (AV binding sites) on their outer surface, making them prothrombotic[90-94]. This is however not necessarily true of all MPs, and partly depends on the nature of the stimulus leading to MP release. EMPs released from activated endothelial cells have a different lipid membrane composition to EMPs released from apoptotic endothelial cells - PS is preferentially expressed on EMPs derived from apoptotic endothelial cells[73, 95], while EMPs derived from activated endothelial cells instead mainly express CD62E on their surface[96, 97].

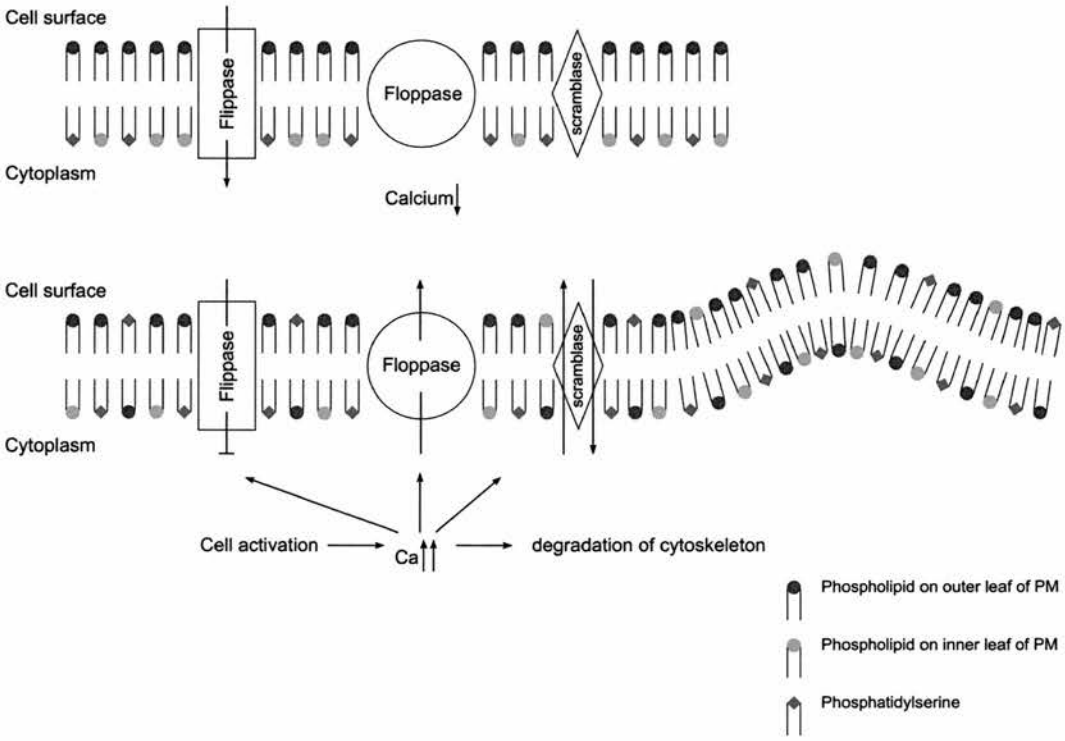


Figure 1.1: Schematic diagram depicting the release of microparticles (MPs). In the steady state, the cellular membrane is asymmetrical with phosphatidylcholine and sphingomyelin found mainly in the outer layer, and phosphatidylserine (PS) and phosphatidylethanolamine (PE) being present in the inner layer. The enzymes flippase, floppase and scramblase are responsible for maintaining this membrane asymmetry. During cell activation or apoptosis, the endoplasmic reticulum releases calcium. This inactivates flippase and activates floppase and scramblase, leading to a loss of the phospholipid asymmetry of the cell membrane and to disruption between the phospholipids and the cytoskeleton. The calcium also activates calpain and gelsolin which cause disruption of the proteins anchored to the cytoskeleton, leading to membrane budding and MP shedding (microvesiculation)[73, 78, 89]. Such MPs are rich in phosphatidylethanolamine and PS on their outer surface.

1.2.2 Function of MPs

MPs are thought to have many important roles, both physiological and pathological. They are detectable in all individuals, whether healthy or with underlying medical conditions, including patients with malignancy with/without thrombosis. It is generally thought that MPs express membrane antigens specific to their cell of origin, and function similarly to their parental cells[78]. These cell-specific antigens are used to identify the subtypes of MPs present in circulation. However it is important to realise that the presence of a particular cell-specific antigen on a MP may not always identify its cell of origin.

It is postulated that soluble antigens circulating in blood derived from a specific cell type may adhere to MPs derived from another cell type, or that MPs derived from one cell type may fuse with the membrane of a different cell type. These cell may theoretically subsequently release MPs carrying an "adopted" antigen[98]. Caution is therefore required when interpreting the results of immunophenotyping experiments. Equally the functional properties and biological role of MPs may differ from that of their cell of origin[99].

Circulating MPs binding to cells may potentially alter the biological activity of the recipient cell, either by 1) transferring receptors that can induce cell signalling or even transformation[99], or 2) by transferring genetic information (mRNA) and second messengers[100]. MPs were shown to transfer mRNA coding for CD81 between leukocytes[101] and TMPs were able to transfer mRNA to monocytes[102]). Such binding of MP surface antigens to their specific counter-receptor on cells allows intercellular signalling, sometimes between far removed cells, as well as other processes, such as regulation of apoptosis, modulation of the immune response, inflammation, angiogenesis and coagulation[98, 99]. As these properties of MPs are better understood, measuring specific markers on MPs or MP dependent activity, rather than the total numbers of MPs, may allow new insights into the underlying pathophysiology of diseases.

Increased levels of circulating MPs have been reported in various medical conditions, including malignancy, inflammatory conditions and thrombosis, suggesting that MPs are true pathogenic markers and possibly effectors of different disease processes[36, 78]. The number, cellular origin, composition and functional properties of the circulating MPs are postulated to reflect the underlying disease[99].

To date the main clinical application of MP analysis has been the simple correlation of MP level with various disease states, i.e. they serve as general indicators of cell injury, stress, thrombosis or inflammation[87, 103]. It is likely that as the measurement of MPs becomes standardised (see Chapter II), the results may be used for clinical intervention. For example, it is well established that many inflammatory conditions are associated with a thrombotic tendency, and it may be that intercellular signalling by MPs is responsible for part of the interaction between the inflammatory and the coagulation systems. MPs induced by *in vivo* stimulation of healthy individuals with a chemotactic peptide induced the release of IL-6 and TF expression by endothelial cells *in vitro*. This was associated with a TF dependent increase in the procoagulant activity of the endothelial cells, an effect which appeared to be mediated by LMPs[104]. There are also a number of studies that have shown increased levels of circulating TF+ve MPs, or increased procoagulant TF+ve MP activity in patients with malignancy +/- thrombosis[18, 81]. It may be that measurement of TF-bearing MPs, or MP-dependent procoagulant function, may help in assessing the risk of thrombosis in patients with various different medical conditions. If patients demonstrate a tendency to increased generation of total and TF+ve MPs, and/or MP-dependent procoagulant activity, targeted therapy to block MP generation or function, could in the future be developed to lower the thrombotic risk.

The expression of certain surface antigens (on parental cells and subsequently on MPs) depends on the cause of cell stimulation. Platelet and endothelial cell stimulation leads to the rapid up-regulation of P-selectin (CD62P) on their surface. The expression of ICAM-1 (CD54) is dramatically up-regulated by pro-inflammatory cytokines so that the presence of CD54+ve MPs indicates inflammatory stimulation

of leucocytes or endothelial cells[98, 105, 106] (ICAM-1 belongs to the immunoglobulin gene super-family of receptors and is constitutively expressed at low levels on endothelial cells, leucocytes, fibroblasts, and epithelial cells). E-selectin (CD62E) or VCAM-1 (CD106) are both expressed on endothelial cells (and therefore EMPs) after stimulation by pro-inflammatory cytokines[95, 98, 107, 108], while apoptotic stimuli of endothelial cells results in EMPs with higher levels of PS and of constitutive endothelial cell markers such as CD31 (PECAM)[95]. The presence of these antigens on the released MPs may determine their functional properties.

There is now good evidence that many "soluble" mediators/markers of inflammation, including adhesion molecules, coagulation factors and cytokines, are actually bound to MPs[36, 87], at least in part, making MPs important circulating bioactive effectors. For example, PECAM-1 (CD31), widely accepted as a soluble marker, can also be detected on PMPs and EMPs by flow cytometry[87] (flow cytometry is unable to detect truly soluble molecules); E-selectin, a frequently measured soluble marker of endothelial stress can also be identified on EMPs by flow cytometry. Similarly, many other "soluble" molecules, such as ICAM-1, VCAM-1, P-selectin, TF, vWF, thrombomodulin and CD40L are now know to be at least partly MP-bound. There is evidence that some of these do exist in true soluble form, usually due to enzymatic cleavage from the cell membrane or by post-translational editing, but there is equally good evidence that a significant proportion (up to 80-90%) occur on cell-derived MPs, presumably with their transmembrane domains intact and normally adjacent proteins present[87]. This is important for two reasons: 1) the release of truly soluble species occurs by entirely different mechanisms from membrane vesiculation, reflecting different pathophysiologies; 2) truly soluble species may have functionally different properties from their MP-bound forms. It is therefore likely that when the MP-bound markers can be clearly distinguished by independent measurement from the truly soluble species, much clearer relationships between the disease state and the marker in question may emerge. This further underlines the importance of standardised MP assays - many of these "soluble" markers are recognised as potentially valuable clinical tools.

1.3 MICROPARTICLES AND MALIGNANCY

There is increasing evidence that MPs are likely to contribute to cancer cell survival, invasiveness and metastases, the latter through tumour micro-environmental degradation and by promoting angiogenesis[99]. MPs also seem to contribute to the ability of cancer cells to avoid apoptosis and escape from immune surveillance – see Figure 1.2, page 20. As evidence of the importance of MPs accumulates, their role in risk stratification and individualization of treatment of cancer patients is likely to become more important. Many different groups have reported increased levels of MPs or MP dependent procoagulant activity in patients with malignancy[99].

1.3.1 MPs and cancer cell survival

It is hypothesised that MPs contribute to cancer cell survival through several different mechanisms. Cells are thought to release MPs as part of a protective mechanism against intracellular stress: intracellular accumulation of caspase 3 normally leads to cell death. If MP release is inhibited, cells accumulate caspase 3 and undergo apoptosis. On the other hand, release of caspase 3-containing MPs allows cell survival[99, 109].

Multidrug resistance of tumour cells has also been linked to the release of TMPs. Chemo-resistant tumour cell lines express more membrane shedding-related genes compared with chemo-sensitive cells[99, 110]. In a study by Safeaei et al[111] MPs released from cisplatin resistant cancer cells contained 2.6 times more cisplatin than MPs released from cisplatin-sensitive cells; in another study the MPs released from tumour cells contained high levels of doxorubicin, so that TMPs seem to contribute to tumour drug resistance[112].

MPs are also likely to protect tumours from complement-mediated lysis: cancer cells can shed MPs containing the complement inhibitor membrane cofactor protein CD46, which promotes the inactivation of complement C4b and C3b[99, 113]. Liberation of CD46 minimises the inflammation in the microenvironment of tumours, protecting the tumour from attack by the complement system[99].

MPs also seem to be involved in suppressing the immune response to tumours, allowing tumour cell proliferation. TMPs from various cancers express Fas ligand, (a ligand of CD95, death receptor Fas) which induces T-cell apoptosis, reducing the adaptive immune response[99]. MPs from lymphoblastoma cells express latent membrane protein-I (LMP-I), another immune suppressing transmembrane protein which inhibits leucocyte proliferation, and which could potentially explain the lack of T-cell proliferation in patients with EBV associated tumours[99]. TMPs have also been shown to fuse with the plasma membrane of monocytes, impairing the ability of the monocytes to differentiate into antigen-presenting cells; and finally tumour cells may also potentially escape the immune system by mimicking the host environment - the tumour cells may fuse with non-tumour cell-derived MPs, thereby receiving phospholipids and membrane-specific proteins that could help them to "hide" from immune surveillance[99]. The immune-suppressive effects of TMPs may facilitate the lymphatic dissemination of malignant cells. Activated platelets, and the PMPs from them, probably also contribute to the haematological dissemination of tumour cells: activated platelets express the adhesion receptor P-selectin while tumour cells express the corresponding P-selectin ligands, such as P-selectin GP and Sialyl Lewis, so that P-selectin bearing platelets and/or PMPs surround the tumour cells protecting them from immune surveillance[99].

1.3.2 MPs and angiogenesis

It is hypothesised that the procoagulant role of MPs also contributes to the survival and dissemination of tumour cells. TF +/- PS on the surface of MPs can initiate and facilitate intravascular coagulation respectively. The fibrin formed coats the tumour cells, protecting them from immune attacks; it also forms a matrix that supports angiogenesis[99]. Thrombin, the final enzyme of the coagulation cascade, cleaves several protease-activated receptors, which in turn can trigger angiogenesis. The cancer cells in turn can potentially also induce angiogenesis by releasing MPs containing mRNA encoding growth factors and by exposure of TF[99]. Activation of the cytoplasmic tail of TF and subsequent downstream signalling can induce angiogenesis[112, 114]. Cancer progression is highly dependent on angiogenesis - the new vessel formation ensures an adequate supply of nutrients, oxygen and growth

factors to the growing tumour and also facilitates tumour dissemination[114, 115]. TF expression on tumour cells correlates with tumour grade and progression, probably reflecting the role of TF in angiogenesis and tumour invasiveness[116-119].

The data on the role of MPs in angiogenesis is however conflicting. PMPs have been reported to be pro-angiogenic, while LMPs and EMPs have been reported as being both pro- and anti-angiogenic. PMPs have been found to stimulate the proliferation of endothelial cells in a dose-dependent manner, and to inhibit their apoptosis[73, 120, 121]. At low concentrations EMPs have been found to be pro-angiogenic as a function of their MMP activity[73, 82] (see later), remodelling of the extracellular matrix through MMP activity being essential for angiogenesis. However at high concentrations EMPs have been reported to be anti-angiogenic as they decrease the formation of capillary-like structures through the production of oxygen free radicals[122]. LMPs have been reported to have either a pro-angiogenic effect through their ability to favour nitric oxide release from endothelial cells[123], or an anti-angiogenic effect secondary to the oxidative stress associated with a reduced release of nitric oxide from endothelial cells[124]. The balance between such pro- and anti-angiogenic factors may partly determine whether harmless in situ tumours progress to frank malignancy, or not[87, 125]. MPs may well be pivotal in this balance.

1.3.3 MPs and cancer cell invasiveness and metastasis

MPs are also likely to enhance tumour invasiveness (and metastasis) by contributing to extracellular matrix degradation, essential for tumour growth[126]. MPs carry proteases, including matrix metalloproteinase (MMP)-2 and MMP-9, and urokinase-type plasminogen activator (uPA). uPA catalyses the conversion of plasminogen into plasmin. Plasmin is a serine protease: it degrades numerous components of the extracellular matrix, including fibrin, and activates the MMP zymogens.

MMPs are a family of zinc-containing endopeptidases whose role in tumour cell invasion, metastasis and angiogenesis has been well established[127]. MMPs have the capacity to degrade all ECM components (basement membrane collagens) as well

as many non-ECM substrates such as chemotactic molecules, latent growth factors, cell-surface receptors, and cell-matrix adhesion molecules. They are mainly secreted in a soluble proenzyme form although some (the membrane-type (MT-)MMPs) are anchored to the cell surface[127]. Of the MT-MMPs, MT1-MMP seems to play a major role in tumour invasion through its ability to activate pro-MMP-2. Of the soluble MMPs, MMP-2 and MMP-9 seem to have a major role in tumour invasion as a result of their ability to degrade type IV collagen (the main component of subendothelial basement membranes).

There are a number of studies that show that MPs and their expression of MMPs are associated with cancer stage and invasiveness. Patients with gynaecological malignancy were found to have higher levels of MPs in their ascitic fluid compared to women with benign causes of ascites[128]. The MPs in the cancer group also differed from the benign group in carrying active MMPs, and the MMP-2 activity of the MPs correlated with the *in vitro* invasiveness of the underlying malignancy[129]. Similarly another group found higher levels of MPs in the ascitic fluid of patients with late-stage ovarian cancer when compared with patients with early-stage ovarian cancer[130]. They also showed that MMP-2, MMP-9 and uPA activity was mainly concentrated within the MPs.

In breast cancer patients, high expression of MMP-2, MMP-9 and MT1-MMP has been correlated with advanced stage disease[127]. The *in vitro* invasive potential of breast cancer cell lines was enhanced by PMPs: PMPs transferred CD41 to the surface of breast cancer cells enhancing their adhesion to endothelial cells and potentially promoting the migration of the tumour cells to the extravascular space[82, 127]; PMPs also stimulated the production of MMPs in invasive breast cancer cells and increased phosphorylation of signaling proteins. Similarly prostate cancer cells, preincubated with PMPs, showed increased invasion potential. This effect was accompanied by an increased secretion of MMP-2[131]. Application of MMP-2/9 inhibitors reversed the PMP-induced tumour cell invasion, so that PMPs seem to promote tumour invasiveness, at least in part, by stimulation of MMP-2 production.

As mentioned previously, MPs are thought to be able to transfer receptors/proteins from one cell type to another, potentially altering the biological activity of the recipient cell. TMPs may therefore contribute to the horizontal propagation of mutant oncogenes such as EGFRvIII, MET, HER-2, allowing cancer growth at distant sites (metastasis)[99]. There is also evidence that TMPs are involved in transferring the genetic information (mRNA) necessary for malignant transformation in between cells.

1.3.4 Potential clinical application of MPs

Further knowledge of the biological functions of MPs may help shape future anti-cancer treatment. Inhibition of MP release is a potential target in anti-cancer treatment since MP release by tumour cells is thought to be associated with improved cancer cell survival and tumour growth through the various mechanisms mentioned above. Some chemotherapeutic agents partly inhibit the mechanism of MP release, e.g. through inhibition of the Rho/Rock pathway; inhibition of the latter pathway resulted in smaller tumour masses in patients with glioblastoma[132].

It is also possible that MPs may in the future be used in the early detection of malignancy. TMPs express tumour-specific markers; Smalley et al[133] compared the MPs in the urine of healthy individuals with the MPs in patients with bladder cancer. The MPs in the cancer patients had higher levels of tumour related proteins, suggesting that the protein composition of such MPs could potentially be used in the early detection of bladder cancer.

MP analysis could potentially provide prognostic information regarding disease progression and response to treatment. In a prospective, nonrandomised single centre study of hormone refractory prostate cancer patients, patients with increased levels of PMPs had a significantly shorter survival[82]. Similarly in other studies (gastric, breast and pancreatic malignancy) patients with higher PMP levels had more extensive disease and a shorter survival[134]. The level of PMPs has been found to be a better predictor of metastasis than other markers such as VEGF, IL-6 and RANTES.

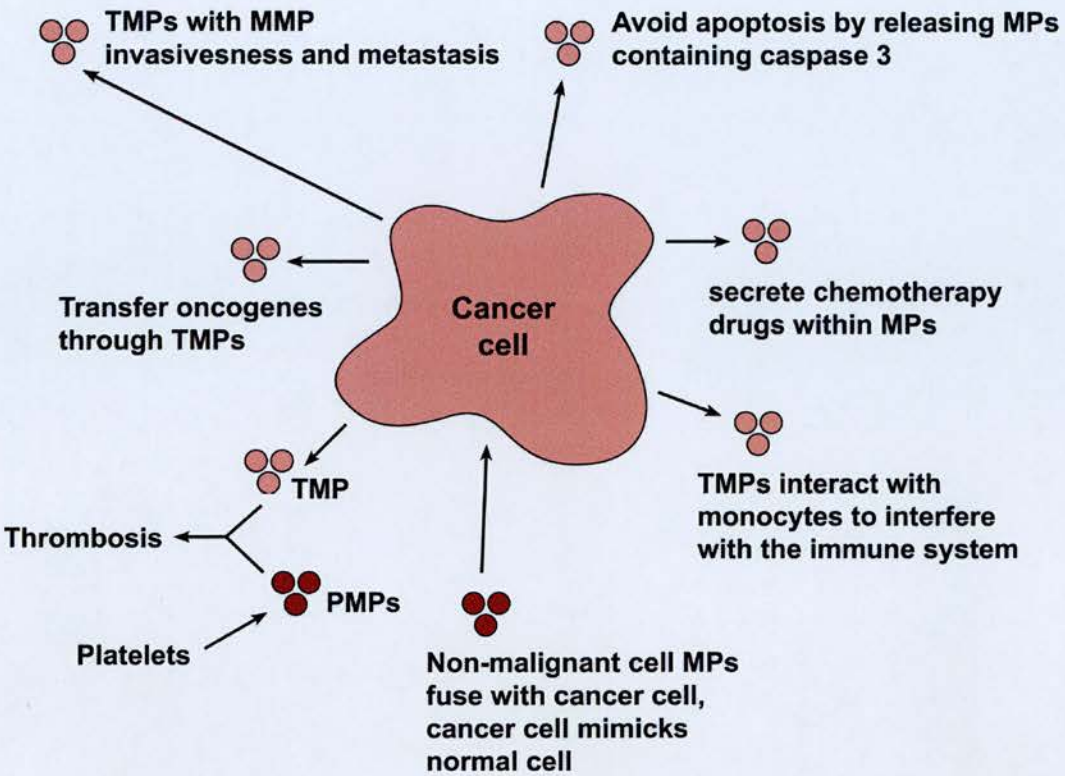


Figure 1.2: The postulated role of microparticles (MPs) in cancer cell survival, invasiveness and metastasis. MPs are thought to allow cancer cells to avoid detection by the immune system, resist the effects of chemotherapy, and avoid apoptosis. MPs are also thought to contribute to the invasiveness of cancer cells and the prothrombotic state of cancer patients.

1.4 MICROPARTICLES and THROMBOSIS in MALIGNANCY

1.4.1 MPs and the clotting system

The specific functions of MPs in normal healthy individuals are still unclear; it is postulated that in health MPs may play an important role in the balance of the haemostatic system, having mainly anticoagulant properties by promoting the generation of low levels of thrombin which activates protein C[98, 135]. The importance of MPs for normal haemostasis is underlined by Scott syndrome[136, 137], a rare autosomal recessive disorder with an inherited defect of the enzyme scramblase which leads to a reduced expression of PS on platelets and reduced release of MPs. As a consequence the ability to assemble the prothrombinase and tenase complexes, and thus activate factor X and prothrombin, is impaired leading to a severe bleeding diathesis. PMPs and other MPs may therefore have an important role in the normal haemostatic response to vascular injury[78].

Under normal physiological conditions the intact endothelial surface has an important anti-thrombotic function. However upon activation endothelial cells become prothrombotic[138]. They release EMPs expressing TF. Such EMPs (and other MP subtypes) provide both a source of TF, as well as a catalytic surface (anionic surface phospholipids) for the assembly of the prothrombinase and tenase complexes[139], thus probably playing an active role in the clotting process.

TF is the physiological initiator of blood clotting[140]. It is a glycoprotein normally expressed on the surface of most nonvascular cells, and after appropriate stimulation also expressed on the surface of monocytes, neutrophils and endothelial cells[141, 142]. It is also variably expressed on the surface of tumour cells. TF binds to circulating factor VIIa anchoring the complex to the surface membrane and dramatically increasing its proteolytic activity for factors X and IX[143]. The hypercoagulable state of patients with malignant disease may be partly explained by the over-expression of TF by tumour cells and cells of the tumour microenvironment (i.e. endothelial cells, fibroblasts, and monocytes/macrophages)[144]. MPs have been identified as the main source of circulating functionally active TF[140]. The importance of TF+ve MPs for haemostasis and thrombosis in otherwise healthy

individuals is still a matter of debate, however several groups have now reported increased levels of TF+ve MPs or TF+ve MP-dependent activity in patients with varying prothrombotic conditions, including malignancy[127], although it is still unclear whether TF bearing MPs and thrombosis in cancer patients are causally linked.

The prothrombotic potential of MPs is however not solely dependent on their expression of TF. The protein and phospholipid composition, as well as the orientation of the phospholipids on the surface of MPs, depends not only on their cell of origin but also on the stimulus resulting in MP release. MPs are thought to have potent procoagulant properties in general, being able to passively support coagulation on their negatively charged surface phospholipids (mainly PS). The phospholipids have a catalytic role promoting the assembly of the tenase and prothrombinase enzyme complexes of the clotting system on the MP membrane surface[87, 140, 145]. Their thrombogenic activity varies proportionally with their PS content. This phospholipid surface has traditionally been thought to be provided by activated platelets (previously known as platelet factor 3), but MPs can also support this process, and in contrast to quiescent platelet membranes, the procoagulant activity of PMPs is enriched and retained on the surface[135]. PMPs have approximately 50 to 100-times higher procoagulant properties than an identical surface area of activated platelets[146]. PMPs also carry FV/Va, GPIIb/IIIa, GPIb/IX, are rich in membrane receptors for coagulation factors, and (together with EMPs) carry significant amounts of vWF (MP bound vWF is functionally more active than freely soluble vWF), all of which contribute to their prothrombotic properties[35, 87, 121, 147-149]. MPs can also express TFPI on their surface and it is likely that the balance of procoagulant and anticoagulant mediators on MPs determines their overall prothrombotic potential[35, 150]. It is difficult to sort out the many influences of MPs on the coagulation system *in vivo* owing to its complexity, however greatly elevated levels of circulating MPs are generally thought to have mainly a prothrombotic rather than an antithrombotic effect.

MPs may also be the link between thrombosis and inflammation – both EMPs and PMPs participate in clot formation and also act as vectors of many inflammatory mediators[26]. Complementary functional assays done in parallel with MP identification would help to confirm the prothrombotic and/or the proinflammatory nature of the MPs detected[87].

In mouse models and in *in vitro* studies, MPs were seen to accumulate at the site of developing clot[151]. They have an important role in clot formation through both their TF-dependent and independent pathways[145]. TNFα stimulation of HUVECs resulted in the release of TF+ve EMPs[70]. Addition of increasing concentrations of these EMPs to a coagulation assay shortened the clotting time compared to EMPs from unstimulated HUVECs. The effect was not seen in FVII deficient plasma, suggesting that the procoagulant activity of the EMPs was TF/FVII dependent. In contrast, EMPs induced *in vitro* by cisplatin treatment were found to be highly procoagulant but thrombin generation was not reduced by blocking antibodies to TF or FVII (TF independent)[79, 152].

There is current interest in carrying out interventional studies[153] evaluating the use of MP levels (especially TF+ve MPs) as markers of thrombogenicity, and looking at the appropriateness of primary thromboprophylaxis in patients considered at high risk of thrombosis by virtue of their number of circulating MPs.

1.4.2 MPs in patients with cancer and VTE

The numbers of circulating MPs and MP-dependent functions have been variably reported to be increased in patients with VTE and/or malignancy by many different investigators[23, 73, 81, 121, 140, 154, 155]. The results in the published literature are variable, probably partly reflecting the lack of standardisation of MP assays, although the variability of published results is also likely to reflect the variability of the underlying medical conditions and their treatment (see Chapter II and Table 1.2, page 27).

Of all MP subtypes, PMPs have been most extensively investigated. PMPs are mainly released from activated platelets[135], although some are released by quiescent platelets, by megakaryocytes, or by apoptotic platelets[73]. PMPs are considered to be important for normal haemostasis; however they may also be involved in pathological thromboses – e.g. high fluid shear stress in diseased small arteries partially obstructed by compression or vasospasm may induce activation and aggregation of circulating platelets, with shedding of procoagulant PMPs[78]. Such PMPs would promote acute arterial occlusion by providing and expanding a phospholipid catalytic surface for the clotting cascade and concentrating surface TF. The TF on PMPs is either derived from the platelets themselves or is transferred to platelets (and in turn PMPs) from other cells such as monocytes[156]. Platelets store small amounts of TF in α-granules[157], and quiescent human platelets also contain TF pre-mRNA, which is spliced into mature mRNA in response to platelet activation, leading to de-novo synthesis of full-length TF protein in platelets[158]. Whether the splicing of platelet TF pre-mRNA is altered in patients with malignant tumours leading to increased TF+ve MPs being released is not yet known; further, the presence of TF on circulating MPs does not necessarily mean that the TF is functionally active[156].

Tumour cells themselves are known to over-express TF[34]. There is good clinical and experimental evidence that cancer cells can be an important source of TF+ve MPs[159]. The number of circulating TF+ve MPs dropped significantly in 3 patients with pancreatic carcinoma after radical pancreatectomy[18]; 50% of the TF+ve MPs also co-expressed MUC-1, a tumour marker for pancreatic carcinoma. Other groups[34] have also shown that the level of TF activity on tumour cells correlates with the thrombotic risk in pancreatic cancer patients[116, 160], and that cancer cells in culture shed procoagulant vesicular structures[72]. It may be that the variability of thrombotic events in cancer patients may partly reflect the variability of MP generation from different tumours, as well as the total tumour burden.

Hron et al[81] compared the MP levels in 20 patients with advanced colorectal cancer with the levels in a healthy control group, and found no difference in the

absolute number of circulating MPs (using light-scattering flow cytometry) between the two groups (the level of PMPs and LMPs were very similar). However the number of TF+ve MPs (mainly PMPs) in the cancer group was almost double that in the control group, and was associated with a four-fold increase in D-dimer levels in patients with malignancy. D-dimer, a degradation product of the fibrinolytic system, is a sensitive marker for activation of the clotting system. Their patient group did not have a history or recent episode of VTE, so they postulate that the increased levels of D-dimers in their cancer patients is likely to reflect tumour related coagulation system activation.

Zwicker et al[18] measured TF+ve MPs using impedance flow cytometry and found that 60% of patients with cancer-associated VTE had detectable TF+ve MPs, compared with 27% of patients with cancer but no VTE, a >4-fold increased risk of thrombosis associated with detectable TF+ve MPs. They also compared a group of patients with idiopathic VTE without cancer, with the cancer patients who had acute VTE to determine whether the presence of TF+ve MPs was a feature of VTE or a feature of malignancy. The median number of TF+ve MPs in the cancer-VTE group was significantly greater than in both the idiopathic VTE and cancer-no VTE groups, suggesting that the high prevalence of TF+ve MPs in patients who present with an acute VTE is associated with cancer patients and not VTE alone.

Tesselaar et al[116], using a coagulation based assay, found increased TF activity, but no difference in the absolute number of circulating TF+ve MPs measured by flow cytometry, in PPP from patients with cancer compared with healthy controls. Patients with malignancy and acute VTE had higher levels of MP-TF activity than healthy subjects, cancer patients without VTE and subjects with idiopathic VTE. This observed association between the levels of MP-TF activity and the development of VTE in cancer patients suggests that TF+ve MPs contribute to the development of thrombosis in malignancy.

Haubold et al[159] similarly found significantly increased levels of TF dependent MP functional activity in a group of patients with prostate cancer, which however did

not correlate with the measured plasma TF antigen level (measured by ELISA) or with the total number of TF+ve MPs measured by flow cytometry. Such differences between the results of functional[159] and antigen-based MP assays have also been reported by other investigators (see Table 1.2, page 27). MP-associated TF activity is a quantitative estimate of the concentration of active TF in the MP preparation, which can act as a cofactor of FVIIa in FX activation, while the number of TF+ve MPs is not a quantitative estimate of the active TF concentration, as the number of TF molecules per MP may vary widely. Also, some of the TF antigen detected on MPs using flow cytometry may be inactive but still detectable by the monoclonal antibodies[116]. Similarly TF antigen levels detected by ELISA may not reflect the true level of active TF present in circulation – not all the circulating TF detectable by ELISA is active, and in the presence of high quantities of circulating plasma membrane bound TF on MPs, the TF may not be sufficiently solubilised by an ELISA's detergent, leading to significantly lower antigen levels being detected[159].

At present it is still unclear whether TF+ve MPs and thrombosis in cancer patients are causally linked, especially because MPs may also support clotting through TF-independent pathways. Circulating TF+ve MPs are not unique to cancer patients; high levels have also been identified in a subset of non-cancer controls as well as in individuals with idiopathic VTEs[71, 73, 161-163]. Once MP assays become standardised the relationship between MPs and thrombosis in cancer patients is likely to become clearer. It may be that in the future the release of MPs from activated endothelial cells and platelets may represent a predictive assay or a therapeutic target to prevent and limit thrombus formation.

Study	Patient group	Method	Number of pts	Results
Toth et al 2008[164]	Breast cancer	Light scattering flow cytometry	n=71	Elevated levels of AV+ve MPs in pts with cancer *vs* pts with benign lumps (p=0.001)
Del Conde et al 2007[165]	Lung cancer	Light scattering flow cytometry, ELISA, coagulation assay	n=17	Increased TF antigen, MP events and TF activity in cancer patient
Hron et al 2007[81]	Colorectal cancer	Light scattering flow cytometry	n=40	Total number of MPs similar in pt and control group; TF+ve MP levels twice as high in pt group (p=0.007)
Zwicker et al 2009[18]	Variable cancer diagnosis	Impedance flow cytometry	n=96	More cancer pts than healthy controls had detectable TF+ve MPs (p=0.002)
Tesselaar et al 2007[116]	Breast and pancreatic cancer	Light scattering flow cytometry, coagulation based assay	n=87	Increased AV+ve MPs and TF activity in cancer pts (p<0.004), but no statistical difference in TF+ve MPs between healthy controls and cancer pts
Haubold et al 2009[159]	Prostate cancer	Coagulation based assay, ELISA, light scattering flow cytometry	n=88	Significantly increased TF activity in cancer pts (p<0.001), but the TF antigen levels and TF+ve MP levels did not correlate with TF activity
Langer et al 2008[144]	Variable cancer diagnosis	Coagulation based assay and ELISA	n=16	TF activity significantly increased in cancer pts
Tilley et al 2008[140]	Variable cancer diagnosis	Coagulation based assay	n=43	TF activity significantly increased in cancer pts (p<0.001)
Chirinos et al 2005[23]	VTE with variable underlying diagnosis	Light scattering flow cytometry	n=50	Elevated EMPs in pts with thrombosis (p=0.001), but no difference in PMP levels

Table 1.2: Summary of a number of published microparticle (MP) assay results in various patient groups. Several investigators have reported increased numbers of circulating MPs and/or MP activity. The number of circulating MPs however often does not correlate with the measured MP-dependent activity. Annexin V (AV), tissue factor (TF), platelet-derived MP (PMP), leucocyte-derived MP (LMP), endothelial-cell derived MP (EMP), venous thromboembolism (VTE), patient (pt).

1.5 MARKERS OF HAEMOSTATIC ACTIVATION

1.5.1 Introduction

Markers of thrombin/fibrin formation and fibrinolysis are in widespread use for both clinical and research purposes. The association of cancer and thrombosis is well established, with thrombosis occurring prior to the diagnosis of cancer in a significant proportion of patients. Activation of the clotting system in patients with cancer occurs through a number of mechanisms. Cancer cells can activate the clotting system directly through tumour procoagulant and TF[166-168], thereby generating thrombin; or indirectly by stimulating mononuclear cells to synthesize and express a variety of pro-coagulants[169], with the consecutive activation of clotting factors leading to increased concentrations of thrombin and fibrin and therefore the liberation of the corresponding markers of haemostatic activation, e.g. D-dimers[170-174].

It is however still unclear whether the increase of such markers of haemostatic activation reflects a cancer-induced prothrombotic state or whether it is simply an epiphenomenon of balanced activation and inhibition of the haemostatic system in cancer patients. Evidence of clotting system activation in cancer patients[20, 175-177] includes a high platelet count, shortened APTT, increased levels of clotting factors (fibrinogen, factors V, VIII, IX and XI), high D-dimers and increased fibrinogen turnover[142]. More recently newer markers of haemostatic system activation, TAT complex and PF1&2, have been shown to be significantly higher in patients with both cancer and DVT than in patients with cancer but no thrombosis or patients with thrombosis but no cancer[20, 142, 178].

1.5.2 D-dimers

D-dimer is a stable degradation product of cross-linked fibrin and reflects the body fibrin concentration. It is a very useful haemostatic marker that can gauge ongoing fibrin degradation[20, 175, 179, 180]. Increased levels of D-dimers in plasma suggest that fibrinolysis has been activated and that excessive levels of fibrin have been produced. Fibrin is also important for tumour metastasis[181-183]; the

formation of a fibrin matrix appears to facilitate tumour growth via the promotion of angiogenesis, and also shields tumour cells from attack by immunocompetent cells[166, 184]. Increased levels of D-dimers have been correlated with tumour extent and associated with a poor prognosis[20, 170, 175, 179, 180, 185, 186].

High D-dimer levels have been associated with VTE in a population based cohort study[187, 188] and prospective studies have shown that D-dimer levels are predictive of thrombotic recurrence in patients with no cancer[189, 190]. Several studies have reported increased levels of D-dimers prior to any treatment in patients with gynaecological malignancy[191]. Satoh reported that increased levels of D-dimer preoperatively were associated with the presence of clinically silent VTE, with a positive predictive value of 36-44%, and a negative predictive value of 89-100%[8]. However D-dimer levels increase in the presence of several different medical conditions including tumours, advanced age, trauma, infection and inflammation[192] so that the main clinical value of measuring D-dimers are for their negative predictive value to exclude VTE[170, 193]. The level of D-dimers has not been found to be particularly helpful in predicting the risk of thrombosis in an individual patient.

1.5.3 Thrombin-Antithrombin Complex and Prothrombin Fragment 1&2

The main event in blood clotting is the production of thrombin from prothrombin[194]. Thrombin is thought to help promote cancer growth and spread via an increase in tumour cell adhesion and by affecting angiogenesis.

Prothrombin Fragment 1&2 (PF1&2) is released when activated factor X cleaves prothrombin to thrombin; the level of circulating PF1&2 reflects the *in vivo* generation of thrombin. Once thrombin is produced it may either act on its substrates (e.g. fibrinogen, factor V, factor VIII), or it may be inhibited by its natural antagonist antithrombin[194] through the formation of a stable inactive enzyme-inhibitor complex, thrombin-antithrombin (TAT). Measuring the level of circulating PF1&2 and TAT is therefore an indirect measurement of the level of activation of the clotting system[166, 194].

Elevated levels of PF1&2 and TAT have been investigated as a risk factor for first and recurrent thrombotic events but studies have reported conflicting results[187, 195, 196]; compared to D-dimers the levels of TAT and PF1&2 have been found to be far less sensitive and provide a lower negative predictive value (78-85%) for the diagnosis of VTE. Measuring these markers of haemostatic activation has not been found to be clinically useful as predictors of VTE in individual patients with malignancy[142]. In a preliminary study, high TAT levels successfully identified patients at higher risk of postoperative VTE, but the positive predictive value was only 23.3%, and these findings were not confirmed in a subsequent study[142]. Similarly neither the PF1&2 nor TAT were predictive of recurrent VTE events in cancer patients treated with oral anticoagulation[142].

1.5.4 Clinical use of the markers of haemostatic activation

Ay et al[187] have found that increased levels of D-dimers and PF1&2 independently predict the occurrence of VTE in patients with cancer; however Beer et al[170, 173, 186, 197, 198] and several other investigators have found that while the markers of haemostatic system activation are increased in patients with active tumours, the increased levels of these markers (D-dimers, TAT and PF1&2) are more useful in assessing prognosis[166, 170] and not helpful in predicting the individual risk of VTE[170-174, 197]. High levels of these markers of haemostatic activation, which reflect the global activation of coagulation and fibrinolysis, have been reported in patients with malignancies both in the presence and absence of thrombosis[187, 199-202].

1.5.5 MPs and markers of haemostatic activation

A number of investigators have measured both the number of circulating MPs and the level of markers of haemostatic activation (D-dimers, TAT, PF1&2) in a variety of different patient groups. Hron et al[81] found a correlation between the number of circulating TF+ve PMPs and the level of D-dimers; however several other investigators have failed to show such a correlation[79, 140]. This variability in results may be due to the use of different methods to analyse MPs. Given the postulated role MPs play in clotting activation and propagation it would be reasonable to assume that there would be a correlation between the two. The current variability in results with most investigators failing to show a correlation is likely to partly reflect the lack of standardisation of MP assays. Once MP analysis becomes standardised it may well be that a correlation between the two will be detectable; it may also be that measuring MP-dependent activity rather than MP numbers would better reflect the role of MPs in coagulation activation. The relationship between MPs (and/or their function) and the markers of haemostatic activation will need to be re-assessed once the assays for MP analysis become standardised

1.6 STUDY HYPOTHESIS, AIMS AND OBJECTIVES

1.6.1 Hypothesis

Gynaecological malignancy is a common diagnosis with a significant proportion of patients surviving for many years. Thrombosis remains a complication with significant associated morbidity and mortality. Thromboprophylaxis is known to reduce the incidence of thrombosis; however thromboprophylaxis in cancer patients is associated with a significant risk of bleeding. It is currently unclear which subgroup of patients with gynaecological malignancy would benefit the most from primary thromboprophylaxis outside the surgical setting. The ability to identify patients at a high risk of thrombosis would allow primary thromboprophylaxis to be targeted to such patients, avoiding the use of thromboprophylaxis in patients at a low risk of thrombosis. This would theoretically reduce the incidence of thrombosis in patients at a high risk of thrombosis, while reducing the bleeding risk for patients at a low risk of thrombosis. Unfortunately at present we are unable to identify such patient subgroups.

MPs are ubiquitous, and are considered to have an important role to play in haemostasis and thrombosis. The number of circulating MPs has been reported to be increased in several patient groups with an underlying prothrombotic potential, including malignancy. It may be that measuring MPs would allow the thrombotic risk of an individual to be better assessed, potentially allowing targeted primary thromboprophylaxis in patients considered to be at high risk of thrombosis.

I hypothesized that patients with gynaecological malignancy have increased numbers of circulating MPs compared to women with no underlying malignancy. I wanted to test this hypothesis and to establish whether measuring the number of MPs in such women would allow us to identify patients at a significantly increased risk of developing a thrombosis. To my knowledge there are no published studies specifically reporting on the number of MPs in patients with gynaecological malignancy.

1.6.2 Aims and Objectives:

- **Set up and standardise a MP assay**
  - Carry out a review of currently published methods for MP analysis
  - Standardise blood sample collection and pre-analytical handling
  - Set up a method to identify and quantify MPs
  - Establish the effect of a freeze/thaw cycle on the number of MPs measured

- **Measure the number of circulating MPs in patients with gynaecological malignancy and in a control group**
  - Recruit women with confirmed gynaecological malignancy (malignant group) and a control group of women with no underlying malignancy to measure the number of circulating MPs
  - Measure the number of circulating PMPs, EMPs, LMPs, TF+ve MPs and AV+ve MPs, and compare the results between the malignant group and the control group
  - Establish the effect of surgery and other treatment modalities on the number of circulating MPs
  - Correlate the number of MPs with the incidence of thrombosis

- **Measure the level of D-dimers, TAT and PF1&2 in the malignant and control groups**
  - Use ELISA to measure D-dimers, TAT and PF1&2 in the malignant group and control group as markers of haemostatic activation
  - Correlate results with numbers of MPs and with the incidence of thrombosis

❖ **Describe the population of patients diagnosed with gynaecological malignancy in the South East of Scotland Cancer Network (SCAN)**

- Identify and describe the demographics of all women presenting with a gynaecological malignancy in SCAN during the same time period as patient recruitment to the study on MPs is carried out

- Establish whether the patient cohort in the MP study is representative of the whole population of patients with gynaecological malignancy in SCAN

- Establish the incidence of the different gynaecological tumours in SCAN, and the incidence of thrombosis in this patient group, and compare to published studies

CHAPTER II

MICROPARTICLE ASSAYS
THE BACKGROUND

2.1 INTRODUCTION

There is currently general agreement on the importance of MPs, both as markers of disease and also as effectors of patho-physiological processes. However, there is still no universally agreed definition of the criteria that define a MP, and there are no standardised methods of sample collection, preparation or method of identification and quantification of MPs.

MPs are described as intact phospholipids vesicles 0.1-1.0 μm in diameter expressing surface antigens of their cell of origin[36, 87, 203]. Some investigators also use AV positivity as a defining feature of MPs[78]; the latter can lead to significant differences in the number of MPs identified as explained later.

The published assay methods for MP analysis are very variable[71], making the comparison of results from different laboratories very difficult. The reported levels of MPs vary significantly[36, 77, 78, 204], in some cases by as much as 3 to 4 orders of magnitude. For example, Tesselaar et al[116], using light-scattering flow cytometry, reported a median of 460 TF+ve MPs per μL of PPP, while Zwicker et al[18], using impedance flow cytometry, reported TF+ve MPs of the order of 70,000 to 3,200,000/μL of PPP. While these differences may reflect the pathology of underlying medical conditions, variations in methodology used for MP measurement, and/or variable sample preparation are likely to be having a significant effect on the observed differences. There is a need to standardize the definition of what constitutes a MP and to standardize sample preparation and the assay protocols used to identify and measure MPs, in order to allow inter-laboratory comparison and meaningful and reproducible interpretation of absolute numbers of MPs.

2.2 PRE-ANALYTICAL VARIABLES

The pre-analytical methods used in obtaining and handling patient samples can significantly influence the number of MPs detected[71, 78] due to the possible *in vitro* activation of and MP release from platelets and other cells[121, 205], which would invalidate the results obtained. The details of the pre-analytical processing of samples need to be taken into consideration when comparing the results from different laboratories.

2.2.1 Needle gauge

There seems to be no difference in the number of MPs detected when using either a 19-gauge needle or a 21-gauge needle (two samples taken concurrently from different arms in the same individual using minimal venous stasis)[78]. However, smaller gauge needles should be avoided, and only minimal venous stasis applied, when taking blood samples to avoid the *in vitro* activation of platelets and subsequent MP release as the blood is collected[206].

2.2.2 Anticoagulant

The effect of different types of anticoagulants on the number of MPs measured has also been compared[78] (0.32% sodium citrate, 10% citric acid-sodium citrate-dextrose (ACD), 17 units/ml sodium heparin, and 75 μM Phe-Pro-Arg-chloromethylketone (PPACK)). Anticoagulants that preserve extracellular calcium (heparin, PPACK) allow continuing *in vitro* microvesiculation and should therefore be avoided when measuring MPs. Most investigators measure MPs in citrated samples; the citrate chelates extracellular calcium limiting *in vitro* microvesiculation. A few groups[98] use ACD, which also chelates extracellular calcium.

2.2.3 Sample processing

MP analysis can be done either directly on whole blood samples[73, 207] or on plasma samples. Using whole blood has the advantage of leaving the MPs in a physiological environment, without any pre-analytical processing steps. However, such samples would need to be analysed as soon as possible to minimise the *in vitro*

production of MPs from blood cells. Paraformaldehyde fixation is not possible due to its effect on cell membranes (causes translocation of PS to the plasma membrane outer layer)[208]. Most investigators instead measure MPs in PFP. This however is a major source of variability due to the many and very different centrifugation protocols in use to prepare PFP[11, 87, 209], either based on serial centrifugation of citrated whole blood or using ultracentrifugation and re-suspension of the resultant MP pellet.

Depending on the centrifugation protocol used there is the possibility of either a loss of MPs (in the discarded sediment with blood cells and platelets, or in the supernatant if MPs are sedimented), or activation of platelets and the *in vitro* generation of MPs. Ultracentrifugation tends to avoid any platelet contamination, however no amount of centrifugation will sediment 100% of MPs because some MPs are no more dense than plasma[87]. There are also concerns that the use of ultracentrifugation results in the generation of MPs *in vitro*. The challenge of accurately measuring the total number of circulating MPs is therefore still unmet. Table 2.1 (page 40) is a summary of some of the more commonly used centrifugation protocols published in the literature. The preparation of PFP is usually considered an essential step prior to MP measurement and needs to be urgently standardised in order to allow direct comparison of the results from different laboratories.

2.2.4 Sample storage

Another source of variability is whether plasma samples are analysed fresh or after a freeze/thaw cycle. Limiting MP analysis to fresh samples is highly labour-intensive, makes collaboration between laboratories difficult, decreases laboratory efficiency, and increases sample-to-sample variation in large sample sizes. However on the other hand there are concerns that the process of freezing itself generates MPs[73, 210]. During a freeze/thaw cycle a significant number of platelets have their cell membranes destroyed producing cell fragments[71]. If this is extrapolated to MPs, freeze/thaw cycles are likely to cause MP fragmentation, leading to false levels of MPs being detected. Rectenwald[35] showed that snap freezing PPP and storing the plasma for 2 weeks at -70°C consistently increased the total number of MPs by 90%

compared to identical fresh samples, suggesting that snap freezing resulted in fragmentation of MPs. Shah et al[78] also examined the effect of storage temperature and duration on MP detection. They measured MPs by flow cytometry either immediately or after storage at either 4°C or frozen for variable time intervals, and found that the MP levels were significantly lower when samples were not processed immediately, regardless of the duration or temperature of storage. The reason for this is unclear, however it is unlikely to be caused by proteolysis or shedding of cellular markers, since experiments with protease inhibitors failed to prevent the disappearance of MPs in samples stored at -80°C[78].

It is currently still unclear what effect a freeze/thaw cycle has on MPs. If it is not possible to process samples in the desired short time frame and samples are to be frozen prior to analysis it is important that true PFP and not platelet-poor plasma is frozen to limit the generation of MPs from residual platelets as much as possible, although this still does not prevent the theoretical possibility of MP fragmentation. The actual freeze/thaw cycle used may further substantially affect the results of MP analysis. Snap freezing of PFP[98] in liquid nitrogen is recommended. Likewise the process of thawing is as important: some investigators thaw MP samples on wet ice[109]; others do a quick thaw at 37°C[98]. The latter should prevent intermediate formation of large ice crystals, however prolonged incubation of a sample at 37°C leads to the deterioration of MPs and the degradation of sensitive antigens. There is no clear best method for thawing the plasma samples to ensure reliable MP results. For each study it is important to establish how MPs of specific phenotypes of interest are affected by a single freeze-thaw cycle. The freezing of MPs needs to be investigated further, since the ability to freeze samples before analysis would significantly increase the usefulness of the method for the diagnostic use of MP assays. Until this is clarified however, MP analysis should probably be done on fresh samples.

Published studies	Method of centrifugation	Sample analysis
Pereira et al 2006[211]	2000*g* x 30mins twice + 20,800*g* x 45mins twice	frozen
Nantakomol et al 2008[203] Combes et at 2004[70] Robert et al 2009[77] Faure et al 2006[212] **This study**	**1500*g* x 15mins + 13,000*g* x 2mins**	**fresh**
Helley et al 2009[82]	2800*g* x 15mins twice	frozen
Choudhury et al 2007[213]	2000*g* x 20mins	fresh
Tesselaar et al 2007[116]	1550*g* x 20mins	frozen
Kim et al 2002[121]	1550*g* x 15mins	fresh
Trummer et al 2008[97]	1500*g* x 20mins	frozen

Table 2.1: There is currently no standardised method of sample storage and preparation for microparticle (MP) analysis. The table above is a summary of some of the different centrifugation protocols in current use to isolate MPs. Different centrifugation protocols are a major source of variability between different laboratories. Different investigators also vary as to whether MP analysis is carried out on fresh platelet free plasma samples or after a freeze thaw cycle, another source of variability.

2.3 TYPES OF ASSAYS

MP assays vary, including: 1) measuring the total number of MPs, 2) measuring MP functional activity and 3) measuring other substances being carried on the MPs. MP analysis is done either by using solid phase assays (functional or ELISA) or flow cytometry, with the latter being used most commonly.

2.3.1 Solid Phase Assays

MPs may be analysed using solid-phase capture functional assays or ELISAs. In functional assays the MPs are first isolated and immobilised from PFP using AV (may underestimate MP levels, see page 47) or monoclonal antibodies to specific cell surface antigens. The functional properties of the MPs are then measured using a prothrombinase assay. Such functional assays give information on MP activity but no details as to the absolute numbers of MPs[36, 73].

Alternatively ELISA methods can be used to identify and quantify MP-bound antigens. ELISAs are much more easily accessible laboratory techniques and can be automated. However they give no information as to the functional properties of the MPs (unlike functional assays) or the absolute numbers of MPs (unlike flow cytometry). They use combinations of antibodies to specific cell surface antigens to allow MP capture and detection in PFP. ELISAs will also detect soluble antigens not bound to MPs (unlike flow cytometry), so that the results from flow cytometry and ELISAs are not interchangeable[135, 214].

2.3.2 Flow cytometry

In flow cytometry, MPs are identified by gating on size (<1 μm) on forward scatter, and fluorochrome positivity for cell specific antigens (using fluorochrome conjugated monoclonal antibodies) – please refer to Figure 2.1, page 44. Flow cytometry allows for the analysis of large numbers of MPs (to the order of tens of thousands), collects information about their size and inner complexity and accurately quantifies the number of circulating MPs (unlike ELISA and functional assays). It is a relatively easy technique to carry out. However many clinical facilities do not have

easy access to flow cytometers, and must instead rely on more conventional assay methods such as ELISA. Also flow cytometry does not provide any information about the functional properties of the measured MPs. There are now a number of literature publications in which there was no correlation between the MP functional assay results, the levels of antigen measured by ELISA, and the number of MPs identified by flow cytometry in the same patient[116, 159]. This is due to the fact that MP numbers identified by flow cytometry do not give any information as to the concentration of specific antigens on the individual MPs, or whether these antigens (e.g. TF) being detected are active or not. It may well be that functional assays are a better reflection of the patho-physiological effect of MPs, although the different techniques are likely to be complementary.

Flow cytometry is the technique most commonly used to analyse MPs. However there are a number of technical difficulties when using light-scattering flow cytometry to identify MPs. The small size of MPs makes their accurate identification difficult. Flow cytometers were designed to analyse cells many orders of magnitude larger than MPs. When using currently available flow cytometers MPs appear close to the electronic noise of the machine, together with cellular debris. The minimal size of MPs has been defined as 0.1 μm mainly because commonly used flow cytometers are unable to distinguish between smaller particles and electronic noise. The limit of resolution on forward scatter between instrument noise and MPs depends on fine optical adjustments and fluidics and optics cleanliness. This may vary with time. It depends on maintenance for a single instrument, and may be different between different instruments. The upper size limit of MPs has been fixed at approximately 1.0 μm because a single bigger MP would be difficult to distinguish from MP aggregates, platelets or MP-platelet aggregates[78].

To try and overcome some of the current technical limitations, Furie et al[5, 73] have reported on a modified commercially available impedance flow cytometer. Impedance flow cytometry is based on the Coulter principle[18]: it determines the electronic volume of a particle based on the fact that the electronic volume is proportional to the change in the impedance associated with the displacement of

electrolyte in a flow cell by the particle of interest. Their modifications include: a slower flow speed, electronic enhancement for a better sensitivity and less noise, optimization of fluid management and ultrafiltration of buffers. With these changes they were able to measure 520 nm beads with an accuracy of +/- 20 nm. It is likely that as the technology advances the accurate identification and quantification of MPs will become easier and more reproducible. Other techniques currently being trialled include the use of thinner laser beams[73], digital-acquiring flow cytometers, dynamic light scattering[215] and electrochemical impedance spectroscopy[216].

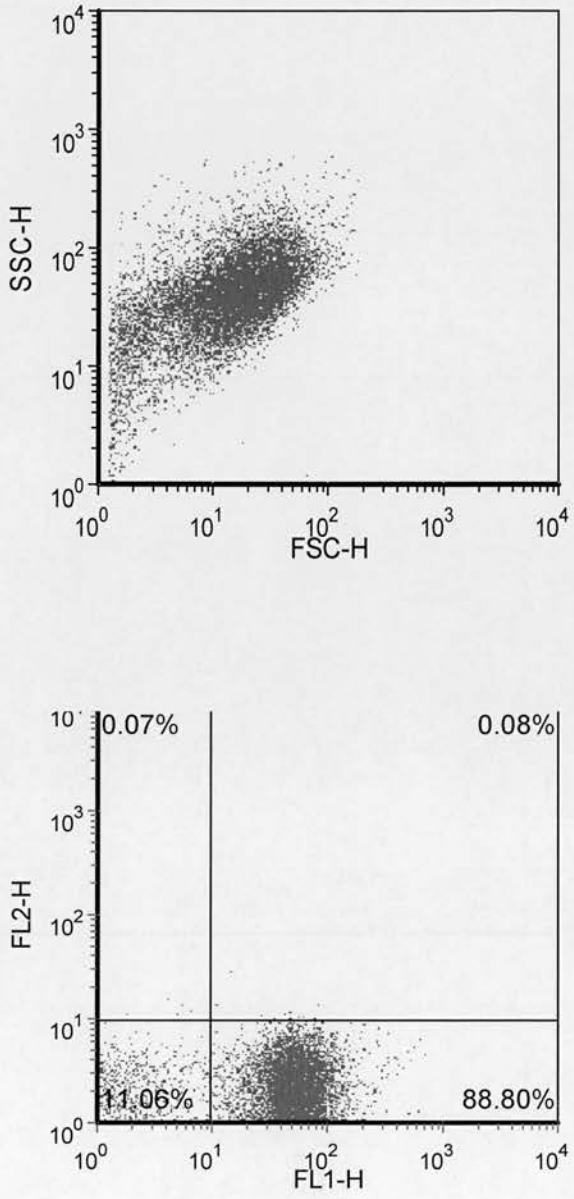


Figure 2.1: Flow cytometer dot plots: Forward scatter (FSC-H) *v.s.* side scatter (SSC-H) dot plot (top image) showing all events <1 μm in size. Microparticles (MPs) are further characterised by immunofluorescence with cell specific monoclonal antibodies. For example in the lower dot plot platelet-derived MPs are being identified by positivity with anti-CD41 FITC (in FL1-H); 1 μm size gate is being applied so that only events <1 μm that are also positive for anti-CD41 FITC are being measured.

2.3.2.1 Sample labelling

Cell specific monoclonal antibodies are used to identify specific subtypes of MPs. When detecting MPs by flow cytometry there are several requirements for target antigens: cell specificity, an abundance of the antigen on the MPs, stability of the antigen, and commercial availability of avid antibodies (preferably monoclonal) that are conjugated to a fluorochrome. There are a wide range of antibodies that can be (and are) used to label MPs prior to flow cytometry to determine their cell of origin[97, 98]. Table 2.2 (page 46) is a summary of some of the commonly used antibodies.

The use of different antibodies to identify the same cell-specific MPs in an individual often results in different quantities of MPs being identified by the different antibodies[98]. MPs originating from the same cell type express different surface antigens according to the stimulus that led to their release. For example CD41+ve PMP and CD42+ve PMP populations are not identical and reflect different pathophysiological phenomena[78, 87, 121]. Different antigens are also likely to be expressed at different concentrations, again leading to variability in their detection.

MP cell of origin	Antigen	Alternative name of antigen
Red blood cell	CD235a	Glycophorin A
Leucocytes	**CD45**	Leucocyte common antigen
Monocyte	CD14	
Granulocyte	CD66b	
T-helper lymphocyte	CD4	
T-killer lymphocyte	CD8	
B lymphocyte	CD20	
Platelets	**CD41**	GPIIb
	CD41a	GPIIb/IIIA
	CD42a	GPIX
	CD42b	GPIbα
	CD61	GPIIIa
	CD62P	P-selectin
Endothelial cells	CD31	PECAM-1
	CD34	Gp105-120
	CD62E	E-selectin
	CD51	αv integrin
	CD105	Endoglin
	CD144	VE-cadherin (most specific for EMPs)
	CD146	S-Endo/Muc 18

Table 2.2: Summary of commonly used antigens to identify the different subtypes of microparticles (MPs) according to their specific cell of origin[98]. The antigens shown in bold have been used in this study to identify LMPs, PMPs and EMPs.

Some investigators include positivity for AV in their definition of what constitutes a MP (besides gating on size and positivity for cell-specific antigens), and only analyze MPs that are able to bind AV. AV binds to PS, and as mentioned in Chapter I not all MPs express PS on their outer surface, this being dependent on the nature of the stimulus leading to MP release. Several studies have now shown that AV negative MPs can make up a significant proportion of all circulating MPs, and in some individuals the concentration of AV negative MPs is 30 times higher than the concentration of MPs that can be detected by AV[73, 87]. Analysing only AV positive MPs results in a significant population of MPs, particularly of endothelial origin, being missed from the analysis[97, 98]. This needs to be taken into consideration when comparing the results from different investigators. Various methods using lipophilic fluorescent dyes, chromophore-labeled lectins, or antibodies to ubiquitous antigens to try and identify the total number of MPs independent of their cell of origin or stimulus leading to MP release have been unable to provide satisfactory results to resolve this issue. At present the use of different antibodies by different investigators to define the same population of MPs is likely to lead to variable results. There is no current method available for the identification of all MPs (irrespective of cell of origin) that allows us to calculate the total number of MPs accurately.

Another source of variability is whether washing steps are included before and after immunolabelling of MPs with monoclonal antibodies. Including washing steps may increase the specificity and minimize the formation of artifactual immunocomplexes; however there is also the risk of losing MPs during several washing steps if this is not done carefully. Such a protocol would require an experienced operator and is time consuming. Several investigators use direct immunolabelling of plasma and flow cytometry analysis without isolation and washing of MPs[217-219]. This method has shown very promising results and would be very useful for clinical diagnostic purposes.

2.3.3 Quantification of MPs

When using flow cytometry, the flow cytometer counts the number of events of a particular fluorescence that pass through its detector. The events of interest are then normally expressed as a percentage of the total number of cells present, allowing their quantification. This however is not possible when PFP is used to identify MPs due to the lack of cells in the PFP. In order to convert the number of events counted into the absolute concentration of MPs in the patients' circulation most investigators use commercially available fluorescent beads at predefined concentration as reference standards. There are two ways of using such fluorescent beads: 1) commercially available fluorescent counting beads of known concentration are added to each patient plasma sample, or 2) the fluorescent beads are used to calculate the volume (x) of sample analysed by the flow cytometer over a pre-defined collection time – this value (x) is then used to calculate the MP concentration when the PFP sample is analysed for the same length of time (flow based method).

In the first method, Flow-Count fluorospheres[121] (Beckman Coulter, Miami, Florida, USA, supplied as a 10 μm-sized bead suspension of a known concentration), or TRUCOUNT™ tubes[203] (Becton Dickinson, San Jose, California, USA, the tubes contain a pre-dispensed lyophilized pellet of a known number of 4.2 μm fluorescent beads) are used. The patient sample (with unknown numbers of MPs) is added to one of the above, and the relative number of MPs detected by the flow cytometer is assessed in comparison to the TRUCOUNT™ tube standard or the Flow-Count fluorospheres. This allows the absolute number of MPs to be calculated[220]. Both TRUCOUNT™ tubes and Flow-Count fluorospheres have been found to be equally reliable[121]. This method is however expensive since every sample has to be added to a TRUCOUNT™ tube, or have Flow-Count fluorospheres added to it.

The alternative and significantly more cost effective method to quantify MPs is to use a flow based method[203]. This method is based on the principle that a specific volume of sample passes through the flow cytometer detector over a set period of time (usually 120 seconds). Knowing the volume of sample that has been analysed

in a given time frame allows the concentration of MPs in the patients' circulation to be calculated. Different investigators[221, 222] have shown that the flow rate through a flow cytometer is constant, so that the volume acquired in a fixed time period will also be constant and can be determined by the use of TRUCOUNT™ beads at the same flow rate as that of sample acquisition. By running a TRUCOUNT™ bead sample at a certain flow rate (the acquisition rate) over a defined period of time (120 seconds), the exact volume that passes through the detector can be easily derived from the number of counts divided by the concentration of TRUCOUNT™ beads, as specified by the manufacturer. This volume is then the reference volume to assess MP concentrations in unknown samples. If the settings regarding flow rate remain the same, the MP count over the same time period (120 seconds) corresponds to the same acquired sample volume, so that the concentration of MPs in the sample can be calculated. The number of events counted in the same acquisition time period is then used to calculate the absolute MP count per μL of test sample. This obviates the need to add reference beads to each sample, and makes it a good method to use for quantification of MPs in serial samples[203]. For accuracy frequent calibration checks should be undertaken at least once at the start and again at the end of each batch of samples to ensure a constant flow rate.

Using a flow rate based method to quantify MPs Storie et al[221] compared the results obtained when using low, medium and high flow rates on the flow cytometer. They found that a high flow rate was associated with the lowest CV and vice versa. They speculate that this is because at the high flow rate, a larger volume is analysed and hence more events are being counted in the 120 second acquisition period. They recommend that a minimum of 100 events should be obtained to ensure reliability, and therefore recommend that high flow rate acquisition is used. Alternatively if a lower flow rate is used longer acquisition times would be necessary to ensure a representative sample is analysed.

2.4 CONCLUSION

There are still many unanswered questions in MP analysis. The current lack of standardization of MP assays means that it is very difficult to compare results in the literature. There is an urgent need to reach an agreement on the methodology of the various MP assays available, including sample handling, instrument settings for flow cytometry and standard markers for quantification e.g. CD144 for EMPs, CD45 for LMPs, CD41 for PMPs, each of defined target epitope (clone) and fluorescence, to allow the reliable comparison of results. It is important to establish the details of pre-analytical handling of samples and sample processing prior to comparing results from different investigators.

Current identification and quantification of MPs is hampered by the limitation of the technology available. Due to their small size it is currently difficult to accurately and specifically identify and quantify MPs. We are still to establish a marker that will satisfactorily identify all MPs irrespective of their cell of origin. However as the technology advances (e.g. dynamic light scattering[215], electrochemical impedance spectroscopy[216]) and the identification of MPs becomes more accurate, MP assays are likely to become clinically relevant in the future.

CHAPTER III

DEVELOPMENT OF MICROPARTICLE ASSAY FOR CURRENT STUDY

3.1 INTRODUCTION

As mentioned in the previous chapter there is currently no standardised method for the identification and quantification of MPs in plasma. However the careful control of experimental procedure would allow comparison between different samples assayed at the same centre. I have devised a flow cytometry based method to identify and quantify the number of circulating MPs derived from platelets, white cells and endothelial cells. I also measured the total number of circulating TF+ve MPs and AV+ve MPs. My method is based on previously published methods with modifications to optimise the identification of the MPs.

3.2 Sample collection

A total of 9 mls of venous blood was collected into three 3.8% trisodium citrate BD Vacutainer tubes (9 parts blood:1 part citrate anticoagulant; Becton Dickinson, San Jose, CA, USA) from an antecubital vein with a 21 gauge needle, using minimal or no venous stasis. All samples were kept at room temperature and processed within two hours of collection to minimize *in vitro* microvesiculation as a result of cell death.

3.3 Microparticle assay

3.3.1 Preparation of PFP

After a review of the literature I compared 3 different centrifugation protocols[77, 97, 116, 203, 212, 220] in order to establish which protocol would be most effective in producing PFP, with minimal platelet contamination – see Table 3.1, page 53. I tested the 3 different centrifugation protocols on blood samples from 5 healthy volunteers. Whole blood was collected into citrated vacutainer tubes (Becton Dickinson, San Jose, California, USA) using a 21 gauge needle with minimal venous stasis. Centrifugation was carried out within two hours at room temperature. In protocol 1 whole blood was centrifuged at 1500*g* for 20 minutes, and the upper two thirds of the supernatant used to measure the residual platelet count (using a Sysmex XE-2100 analyser). In protocols 2 and 3, the supernatant after the first centrifugation step was removed and centrifuged further as shown in Table 3.1, page 53. The upper

two thirds of the supernatant after the second centrifugation step was then used to measure the residual platelet count. Protocol 3 (1500*g* x 15 minutes + 13,000*g* x 2 minutes) consistently produced PFP with a residual platelet count of $5x10^9$/L or less. I therefore used this centrifugation protocol in my study.

In the study cohort the residual platelet count in the PFP was in almost all cases less than $5x10^9$/L, with no residual detectable platelets in approximately half of the samples – please refer to Table 3.2 for details.

Protocol number	**Centrifugation Protocol**	**Residual platelet count ($x10^9$/L)**
1	1500*g* x 20 minutes	40-80
2	1500*g* x 20 minutes x2	1-15
3	**1500*g* x 15 minutes + 13,000*g* x 2 minutes**	**0-5**

Table 3.1: The 3 different centrifugation protocols initially compared, showing the centrifugal force used (*g*) and the duration of centrifugation. Protocol number 3 consistently produced a residual platelet count <$5x10^9$/L, and was the one used in this study.

Number of patients n=109	**Residual platelet count ($x10^9$/L)**
51 (46.7%)	0
51 (46.7%)	1-5
7 (6.4%)	6-9
0	>9

Table 3.2: The residual platelet count in the platelet free plasma of study samples.

3.3.2 Immunophenotyping of MPs

Once the PFP was prepared small aliquots of it were labelled with the different immunofluorescent monoclonal antibodies. As per published methods[82, 97, 121, 203] in the literature 20 μL of PFP were diluted in 100 μL of PBS and incubated with 2 μL of immunofluorescent monoclonal antibody at room temperature for half an hour in the dark. When staining with AV, 20 μL of PFP were diluted in 100 μL of binding buffer (0.1M Hepes, 1.4M NaCl, 25mM $CaCl_2$, pH 7.4, (10x concentrate), BD Biosciences) and incubated with 5 μL of AV as above. Once the incubation period was over PBS or binding buffer respectively was added to make up 1 mL of volume, and the sample analysed on the flow cytometer (see later). For the first twenty patients recruited, aliquots of 500μL of the PFP were also stored frozen at -80°C (see section 3.5, page 64).

3.3.3 Flow cytometer

All samples were analysed using an unmodified BD FACScan flow cytometer using CELL Quest software (Becton Dickinson Immunocytometry Systems, CA, USA). The data generated was analysed using FCS express software (version 3, DeNovo Software). The flow cytometer was cleaned on a daily basis as per the manufacturer's instructions, and calibrated on a regular basis using Calibrite^{TM} beads (BD Biosciences).

3.3.4 Reagents and monoclonal antibodies

The list of antibodies used in this study is summarised in Table 3.3, page 56. I used directly conjugated monoclonal fluorescent antibodies to identify cell-specific MP subtypes. The antibodies I selected for this study are based on published literature[98]. I selected monoclonal antibodies against antigens with high cell specificity. In order to ensure minimal disturbance from non-specific binding of the antibodies, each patient sample was also incubated with non-specific FITC/PE/APC-conjugated anti-mouse IgG1 antibodies, to set a positive *v.s.* negative discrimination limit.

The different subtypes of MPs were defined as events <1 μm in size and positive with the following antibodies:

PMPs positive with anti-CD41,

activated PMPs dual positive with anti-CD41 and anti-CD62P

EMPs positive with anti-CD144

LMPs positive with anti-CD45

TF+ve MPs positive with anti-CD142

AV+ve MPs positive with AV.

All the antibodies were titrated to determine the optimal saturating concentration for MP labelling. I found that the optimal dilution for the different antibodies was as follows:

anti-CD45 PE, anti-CD144 PE, anti-CD142 FITC and AV APC - neat;

anti-CD41 FITC and anti-CD62P PE diluted 1 in 2;

isotype controls diluted 1 in 4.

Examples of the titrations are shown in Figure 3.1, page 57-58.

The flow cytometer used in this study allows multi-colour labelling with up to 4 different colours. I had initially planned to use multi-colour flow cytometry to be able to use combinations of antibodies in order to identify the MP subtypes that were positive for TF and AV. However during my initial experiments I found that the binding buffer, necessary for AV binding, was interfering with the binding of the cell-specific antibodies. Similarly there was interference between anti-CD142 and the other antibodies. I was therefore unable to use these antibodies in combination with other antibodies, so that while I was able to measure the total number of TF+ve (CD142) MPs, and the total number of AV+ve MPs, I was unable to ascertain the cell-specificity of these MPs. There was no adverse interaction between anti-CD41 and anti-CD62P, and these two antibodies were therefore used in combination to further characterise the PMPs identified.

MP subtype	Fluorochrome	Antigen	Company	Clone
PMP	FITC	CD41 (GPIIb)	AbD Serotec	PM6/248
Activated PMP	PE	CD62P (P-selectin)	AbD Serotec	Psel.KO.2.12
EMP	PE	CD144 (VE-Cadherin)	Beckman Coulter	TEA1131
LMP	PE	CD45 (common leucocyte antigen)	BD Biosciences	H130
TF+ve MP	FITC	CD142 (tissue factor)	AbD Serotec	CLB/TF-5
AV+ve MP	APC	phosphatidylserine	BD Biosciences	
Isotype negative controls	FITC PE APC	n/a	BD Biosciences	MOPC-31C MOPC-21 MOPC-21

Table 3.3: List of monoclonal antibodies and isotype negative controls used in this study to label microparticles (MPs).

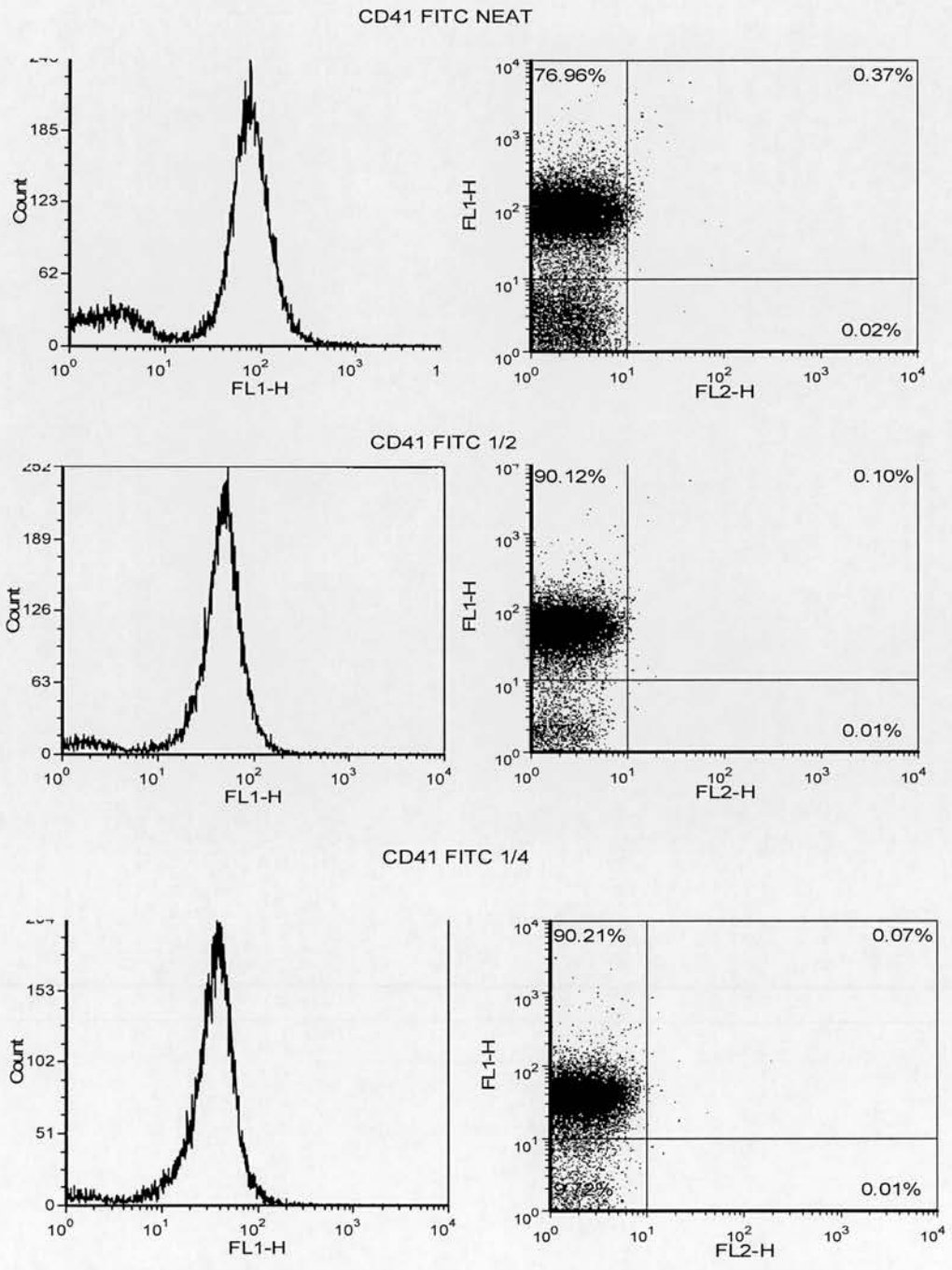


Figure 3.1a: An example of antibody titration using anti-CD41 FITC antibody. The fluorescence histograms (images on the left) show the value for peak channel fluorescence for each dilution; dot plots (images on the right) show anti-CD41 positive events (FL1-H), detecting platelet-derived microparticles. A 1 in 2 dilution allows good separation of CD41 positive events from CD41 negative events.

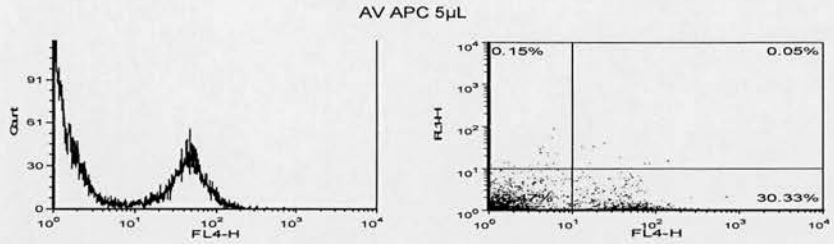


Figure 3.1b: Annexin V (AV) APC neat – fluorescence histogram (image on the left) showing the peak channel fluorescence with AV neat; dot plot (image on the right) showing AV positive microparticles (FL4-H).

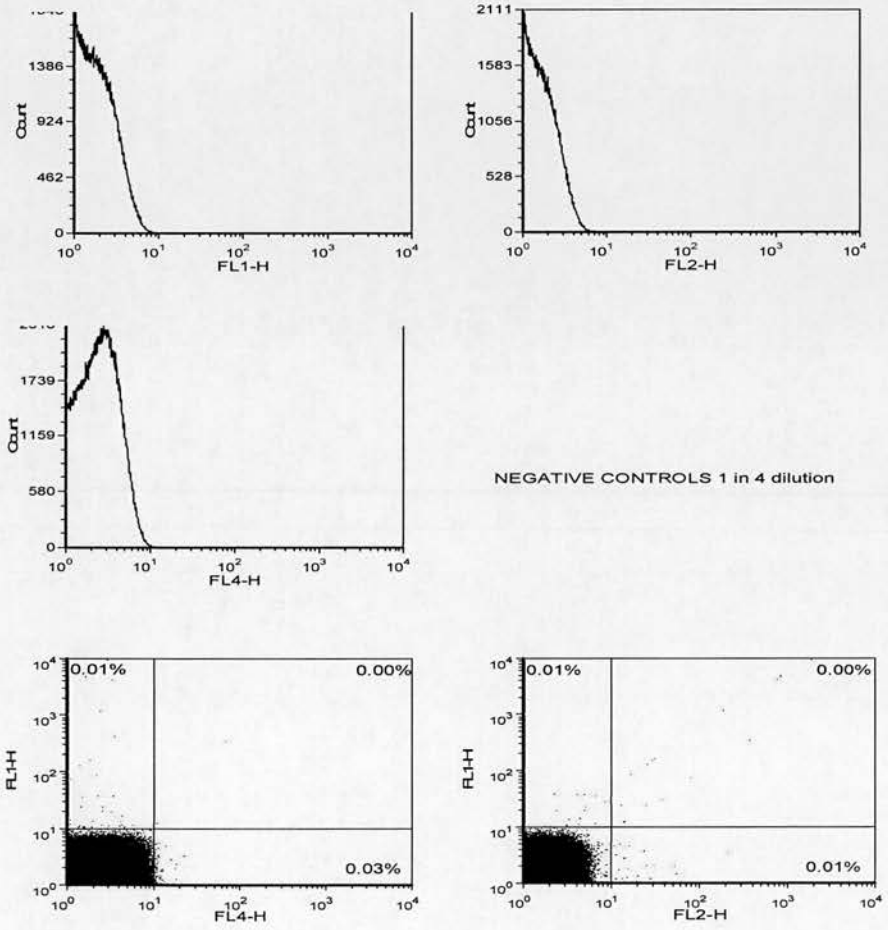


Figure 3.1c: Negative controls 1 in 4 dilution: fluorescence histograms and dot plots to establish the positive and negative gates for each fluorochrome.

3.4 Flow cytometer protocol

3.4.1 Size calibration

The MP size gate was defined using 1 μm diameter latex beads (Duke Scientific Corporation, Palo Alto, CA). These beads were diluted 1:10,000 in 0.5% albumin in PBS. The flow cytometer settings were changed to MP settings (please refer to Figure 3.3, page 60) and the data was acquired on a contour plot after sonication of the 1 μm beads. This data was used to establish the 1 μm gate on log forward and side scatter dot plots, and was subsequently applied to all MP data analysis – see Figure 3.2

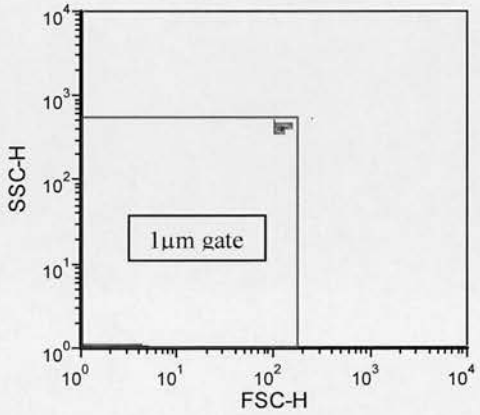


Figure 3.2: 1 μm bead size calibration to set microparticle gate based on size.

The forward scatter and side scatter settings were set at logarithmic gain and the settings were optimised to be able to identify events less than 1 μm in size, at the same time minimising non-specific electronic noise. Fluorescence gates were set by forward gating of samples labelled with monoclonal antibodies, using negative isotype control samples for comparison to exclude non-specific background staining. Please refer to Figure 3.3, page 60 for the flow cytometer settings used. Among the events within the 1 μm gate, MPs were positively identified according to their fluorescence positivity for cell-specific antigens.

Detectors/Amps

Parameter	Detector	Voltage	Amp gain	Mode
P1	FSc	E00	9.99	Log
P2	SSc	350	1.58	Log
P3	FL1	540	1.0	Log
P4	FL2	590	1.0	Log
P7	FL4	600	1.0	Log

Threshold

Primary parameter	Secondary parameter
FSc	none
value 20	n/a

Compensation

FL1	18% FL2
FL2	37.1% FL1
FL2	0% FL3
FL3	18.1% FL2
FL3	1.5% FL4
FL4	5.7% FL3

Figure 3.3: Flow cytometer settings for microparticle analysis.

3.4.2 Quantification of MPs

In order to be able to quantify the number of circulating MPs in the patient plasma I used a flow based method. As per published methods[221] I used TRUCOUNT™ beads (BD Biosciences) of known concentration in order to establish the volume of fluid analysed by the flow cytometer over a set period of time (120 seconds).

TRUCOUNT™ beads are supplied at a specific concentration (approximately 1000 beads/μL). They are 4 μm in diameter, and are labelled with both FITC and PE. 50 μL of such TRUCOUNT™ beads were diluted in 950 μL of PBS, using the recommended reverse pipetting technique. The flow cytometer settings were changed so as to optimise the detection of the TRUCOUNT™ beads according to the advice given by the manufacturer (Figure 3.4, page 62 and Figure 3.5, page 63). The beads were vortexed carefully immediately prior to analysis; a minimum of 10 seconds was allowed before commencing data acquisition to allow stabilization of the sample flow rate. Data was then acquired at medium flow rate for 120 seconds twice prior to and again twice at the end of each batch of patient samples (to ensure a steady flow rate), and the mean bead count was calculated from these 4 sets of data. Knowing the number of events measured by the flow cytometer over 120 seconds, and the concentration of the TRUCOUNT™ beads, allowed me to calculate the volume of fluid analysed by the flow cytometer over 120 seconds. There is published evidence[221] that the flow rate through a flow cytometer is stable, so that the same volume of fluid is analysed in the same time interval at the same flow rate, confirmed also by my results. My mean bead count at medium flow rate over 120 seconds was 3744 beads with a standard deviation of 122 beads over 120 seconds, and an overall inter-assay CV over 12 months of 3.2%.

Each patient sample was then analysed in the same manner as the TRUCOUNT™ beads i.e. for 120 seconds at medium flow rate, but changing the flow cytometer settings to optimise MP identification (Figure 3.3, page 60) – this allowed me to then calculate the concentration of MPs in patient plasma by the calculation shown in Figure 3.6, page 63.

Detectors/Amps

Parameter	Detector	Voltage	Amp gain	Mode
P1	FSc	E00	2.0	Log
P2	SSc	341	1.0	Log
P3	FL1	505	1.0	Log
P4	FL2	597	1.0	Log

Threshold

Primary parameter	Secondary parameter
FL1	none
value 181	n/a

Compensation

FL1	18% FL2
FL2	37.1% FL1
FL2	0% FL3
FL3	18.1% FL2
FL3	1.5% FL4
FL4	5.7% FL3

Figure 3.4: Flow cytometer settings used to analyse TRUCOUNT™ beads.

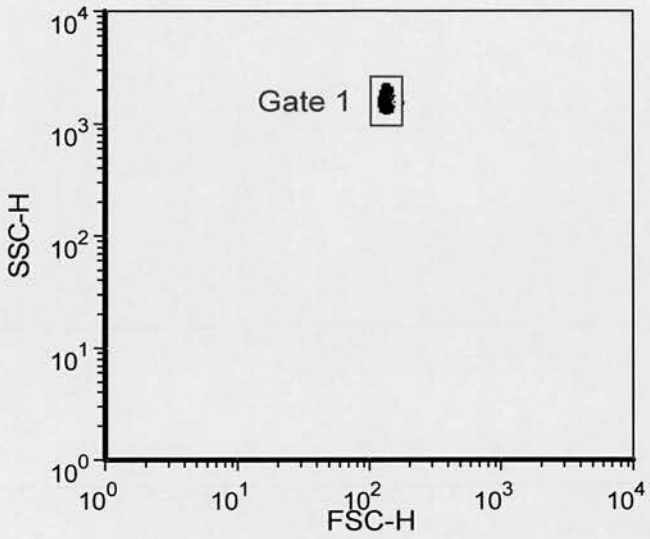


Figure 3.5: Dot plot of TRUCOUNT™ beads.

$$\text{Final TRUCOUNT}^{TM}\text{ concentration (beads/}\mu\text{L)} = \frac{\text{Stated bead concentration (beads/}\mu\text{L)} \times 50\ \mu\text{L}}{1000\ \mu\text{L}}$$

$$\text{Volume sampled in 120 seconds (}\mu\text{L)} = \frac{\text{Mean bead count in 120 seconds}}{\text{Final TRUCOUNT}^{TM}\text{ concentration (beads/}\mu\text{L)}}$$

$$\text{Final sample MP concentration (MP/}\mu\text{L)} = \frac{\text{MP count in 120 seconds}}{\text{Volume sampled in 120 seconds (}\mu\text{L)}}$$

$$\text{Original plasma MP concentration (MP/}\mu\text{L)} = \frac{\text{final sample MP concentration (MP/}\mu\text{L)} \times 1000\mu\text{L}}{20\ \mu\text{L}}$$

Figure 3.6: Method used to calculate the original microparticle (MP) concentration in patient plasma. The above calculation is based on 50 µL of TRUCOUNT™ beads being diluted in 950 µL of phosphate buffered saline (PBS), and 20 µL of patient plasma being diluted to a final volume of 1000 µL of PBS or binding buffer prior to flow cytometer analysis.

3.5 Effect of freezing PFP on MP analysis

Limiting MP analysis to fresh samples is highly labour-intensive and significantly limits the number of samples that can be run. It also makes inter-laboratory collaboration difficult, decreases laboratory efficiency, and increases sample-to-sample variation in large sample sizes. In view of this I assessed whether MP analysis could be done on plasma samples that had been stored frozen until analysis at a later date.

For the first twenty patients, I measured MPs on a fresh and again on a frozen aliquot of the same PFP sample. The fresh sample of PFP was processed routinely. A separate 500 µL aliquot of the same PFP sample was frozen and stored at -80°C until analysis. This frozen sample was then thawed at room temperature, the number of MPs measured as per the usual protocol and the results of the paired PFP patient samples compared.

The two sets of results were significantly and variably different - Figure 3.7, page 65. In the majority of cases the number of MPs detected after a freeze/thaw cycle was greater than on fresh samples, except for EMPs and AV+ve MPs where the converse was true. However for some patient samples the reverse was also true. While the mechanisms behind these changes are difficult to explain, these results suggest that freezing has the potential to introduce significant experimental artefacts. In view of this in the rest of the patients MPs were measured on fresh plasma samples to ensure that the results were comparable.

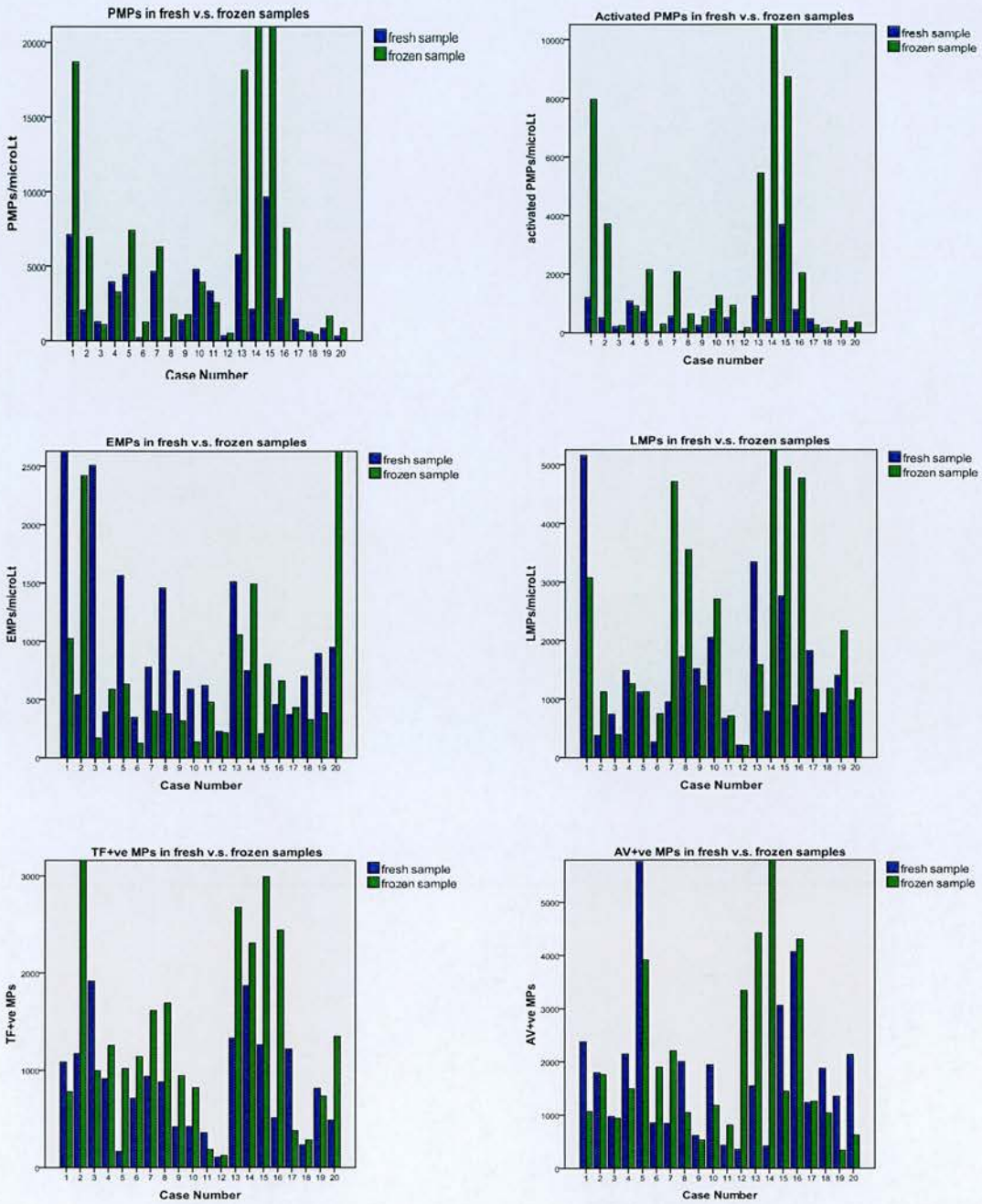


Figure 3.7: Comparison of results between fresh and frozen samples. The number of microparticles detected after a freeze/thaw cycle did not correlate with the results obtained from fresh plasma.

3.6 D-dimers, TAT and PF1&2 Assays

3.6.1 Preparation of PFP

As for the MP analysis all the samples were processed at room temperature within two hours of collection. In order to prepare PFP for the measurement of D-dimers, TAT and PF1&2 one of the citrate tubes from each patient was centrifuged at 2500*g* for 15 minutes, as per our local laboratory standard operating procedure for such assays.

3.6.2 Storage of samples

An aliquot of PFP prepared as above from every patient recruited was stored frozen at -80°C until a later date. At the end of patient recruitment all stored samples were thawed at 37°C as per our local laboratory standard operating procedure and ELISAs carried out.

3.6.3 Assays

The level of D-dimers, TATs and PF1&2 were measured using ELISAs:

- HaemosIL D-Dimer HS, Instrument Laboratory UK Ltd (IL),
- Enzygnost TAT micro, Dade Behring, Marburg GMBH
- Enzygnost f1+2 micro (monoclonal), Dade Behring, Marburg GMBH

3.7 FINAL PROTOCOL USED IN THIS STUDY

Sample Collection and Processing:

Venepuncture with no or minimal venous stasis from an antecubital vein, using a 21-gauge needle.

9 mls of venous blood collected into 3 citrated tubes.

Samples kept at room temperature and processed within a maximum of two hours.

Centrifugation:

a) PFP preparation for MP analysis

Two citrate tubes from each patient were centrifuged at 1500*g* for 15 minutes; the upper two thirds of the supernatant were removed and centrifuged further at 13,000*g* for 2 minutes.

The resultant supernatant (PFP) was used to measure MPs. MP analysis was carried out as soon as the PFP was prepared.

A small aliquot of the PFP was used to measure the residual platelet count in the plasma.

b) PFP preparation for D-dimers, TAT and PF1&2

One citrate tube from each patient was centrifuged at 2500*g* for 15 minutes; the resultant supernatant was stored at -80°C. These samples were later thawed at 37°C and the levels of D-dimers, TAT and PF1&2 measured using ELISAs as the per manufacturer's instructions.

Immunophenotyping:

For each patient sample 20 µL of PFP were diluted in 100 µL of PBS or binding buffer and incubated with directly conjugated immunofluorescent monoclonal antibody or AV as follows:

Tube 1: 20 µL of PFP + 100 µL of PBS + 2 µL anti-mouse IgG1 FITC + 2 µL anti-mouse IgG1 PE + 2 µL anti-mouse IgG1 APC

Tube 2: 20 µL of PFP + 100 µL of PBS + 2 µL anti-CD41 FITC + 2 µL anti-CD62P PE

Tube 3: 20 µL of PFP + 100 µL of PBS + 2 µL anti-CD144 PE

Tube 4: 20 µL of PFP + 100 µL of PBS + 2 µL anti-CD45 PE

Tube 5: 20 µL of PFP + 100 µL of PBS + 2 µL anti-CD142 FITC

Tube 6: 20 µL of PFP + 100 µL of binding buffer + 5 µL of AV APC

The above test tubes were incubated in the dark at room temperature for half an hour.

The antibody dilutions used were as follows:

Isotype negative controls – 1 in 4 dilution

Anti-CD41 FITC, anti-CD62P PE – 1 in 2 dilution

Anti-CD144 PE, anti-CD45 PE, anti-CD142 FITC and AV APC – neat

When more than one patient sample was being analysed on a specific day, antibody labelling of the different patient samples was staggered so that all samples were only incubated for a total of half an hour ensuring uniformity.

Once the incubation period was over PBS or binding buffer was added to each test tube to make up 1 mL of volume as follows:

Tube 1: 874 µL PBS

Tube 2: 876 µL PBS

Tube 3: 878 µL PBS

Tube 4: 878 µL PBS

Tube 5: 878 µL PBS

Tube 6: 875 µL binding buffer

The samples were then immediately analysed on the flow cytometer.

Flow cytometer analysis:

While the patient samples were incubating, the flow cytometer was cleaned as per the manufacturer instructions.

TRUCOUNT™ beads preparation:

50 µL of TRUCOUNT™ beads of known concentration (approximately 1000 beads/µL) were diluted in 950 µL of PBS.

TRUCOUNT™ beads analysis:

The flow cytometer settings were changed to optimise detection of the TRUCOUNT™ beads (see Figure 3.4, page 62), the flow rate set to medium, the beads vortexed and after at least 10 seconds of flow (to allow stabilization of the sample flow rate) the beads were analysed for 120 seconds.

This was done twice prior to each batch of patient samples and twice again at the end of patient sample analysis. The 4 event counts thus generated were used to calculate the mean bead count over 120 seconds. This value was later used to calculate the volume of fluid analysed by the flow cytometer over 120 seconds (see Figure 3.6, page 63).

Patient sample analysis

The flow cytometer settings were then changed to MP settings (Figure 3.3, page 60), the flow rate was kept at medium rate, the patient sample was vortexed, and after at least 10 seconds of flow (to allow stabilisation of the sample flow rate) the patient sample was analysed for 120 seconds.

3.8 DISCUSSION

At the time of writing there are still many unanswered questions about MPs. It is still unclear how best to identify them accurately and specifically. This makes comparison of results between different laboratories very difficult. Different investigators use slightly different criteria to define what constitutes a MP, and there is no currently standardised method to identify them. Accurate identification of MPs is hampered by their small size which challenges the currently available technology. There are various methods by which MPs can be analysed. I have devised a method based on light-scattering flow cytometry.

It is still unclear how best to process samples pre-analysis to ensure no loss or generation of MPs *in vitro* during sample preparation. In order to produce meaningful results and allow inter-patient correlations, I ensured that all the patient samples were treated in an identical manner. All patients were sampled from an antecubital vein with no or minimal venous stasis applied. All samples were kept at room temperature and centrifuged within 2 hours. Incubation was always limited to half an hour and all MP analysis on the flow cytometer was done immediately the PFP was prepared. When more than one patient sample was being analysed on a specific day, immunophenotyping of the plasma was done in a staggered fashion to ensure uniform incubation and handling.

Flow cytometry is currently the commonest technique used to identify MPs. It allows the analysis of tens of thousands of MPs, and the identification of the MP subtype by positivity for cell specific antigens. However flow cytometry has a number of limitations when used to measure MPs. MPs are generally defined as intact phospholipid vesicles 0.1-1.0 μm in diameter[78], so that they are much smaller than the cells which flow cytometers were initially designed to analyse. The MPs appear close to the electronic noise and cellular debris in current flow cytometers making their positive identification challenging. This is a limitation of my study. In this study I defined MPs as events less than 1.0 μm in size that were positive for the cell-specific antigens. I therefore set a 1.0 μm gate to capture all

events 1.0 μm or less in size, minimising interference by any residual contaminating platelets. On the other hand I set the forward scatter threshold at 20 to maximise detection of the smallest MPs present while removing some of the machine noise.

Prior to processing patients samples I trialled out different centrifugation protocols to establish how best to prepare PFP with minimal platelet contamination. I concluded that based on current evidence ultra-centrifugation was probably best avoided due to the real concern of "generating" MPs during the ultra-centrifugation process, and as mentioned in the previous chapter even ultra-centrifugation cannot guarantee that all the MPs are isolated. After reviewing the literature I tried out 3 different centrifugation protocols. Based on my data the first two (please refer to Table 3.1, page 53) did not adequately remove contaminating platelets from the plasma, while protocol number 3 (1500*g* x 15 minutes + 13,000*g* x 2 minutes) produced PFP with a residual platelet count consistently $\leq 5x10^9$/L. This was borne out in my study – none of the patient PFP samples had a platelet count above $9x10^9$/L, 46.7% had no residual contaminating platelets, another 46.7% had a residual platelet count of 1-$5x10^9$/L and only 6.4% of all samples had a platelet count of 6-$9x10^9$/L. This is important because any residual platelets in the plasma sample are likely to interfere with the accurate detection of PMPs.

In order to be able to accurately quantify the absolute number of circulating MPs I made use of a flow based method, using TRUCOUNT™ beads of known concentration to determine the volume of fluid analysed by the flow cytometer over a specific period of time. There are published studies[221] showing that the flow rate through a flow cytometer is constant. I have also confirmed this in my study. The intra-assay variation in the bead count was low with only a small variation in the flow rate over a single flow cytometry session and an overall method inter-assay CV over 12 months of 3.2%. I found this method of quantifying MPs to be reproducible and more cost-effective than if I had added Flow-Count fluorospheres to each patient tube.

There are many different monoclonal antibodies that can be used to identify specific subtypes of MPs. The antibodies I chose to use in my study (please refer to Table 3.3, page 56) were based on published literature[98], and were chosen because of their reported specificity for a particular cell type. Anti-CD41 binds to GPIIb, an antigen normally expressed on all platelets and specific to platelets. CD62P (P-selectin) on the other hand is normally expressed on activated platelets and activated endothelial cells. By using anti-CD62P together with anti-CD41 I was able to further characterise the circulating PMPs (PMPs expressing both CD41 and CD62P being released from activated platelets). CD144, also known as VE-cadherin, is expressed by most endothelial cells. Endothelial cells express many different surface antigens, but CD144 seems to be the surface antigen most specific to endothelial cells, the only other cells expressing CD144 being perineural cells. CD45, also known as the common leucocyte antigen, is expressed on all white cells and is specific to white cells, so that anti-CD45 identifies LMPs. CD142 is the tissue factor antigen, while AV binds to PS. The antibodies I used were titrated to determine their saturating concentrations and optimal dilutions.

I had initially planned to use combinations of up to 4 different antibodies using multicolour flow cytometry to characterise the AV positivity and TF positivity of the different subtypes of MPs. However in the initial experiments I found significant interaction of the AV binding buffer with the other antibodies; anti-CD142 also interfered with the other antibodies leading to a lack of reproducibility. I therefore elected to use all the antibodies singly (other than anti-CD41 and anti-CD62P which did not interfere with each other) in order to ensure reproducible results. This however meant that while I was able to measure the total number of PMPs, EMPs, LMPs, TF+ve MPs and AV+ve MPs, I was unable to comment on the TF positivity or the AV positivity of the PMPs, EMPs and LMPs. I also had no way of establishing the cells of origin of the TF+ve and AV+ve MPs I was detecting.

A significant source of variability in the published literature is whether MP analysis is carried out on fresh or frozen samples, with a number of investigators[82, 97, 116, 211] using frozen samples. This has the great advantage of being able to batch

samples allowing greater efficiency in analysis, while limiting MP analysis to fresh samples is highly labour intensive and makes inter-laboratory cooperation difficult. However it is currently unclear what effect a freeze/thaw cycle has on MPs. In view of this for the first 20 patients I measured the number of circulating MPs both on a fresh sample and a separate aliquot of the same plasma that had been stored at minus 80°C prior to MP analysis. My results on fresh samples compared to the same sample after a freeze/thaw cycle were very variable and significantly different. It is difficult to explain the variability in these results, however it is likely that freezing has the potential to introduce significant experimental artefact. As a result all MP analysis in my study have been carried out on fresh samples within 2 hours of blood collection to ensure that the results are comparable.

3.9 SUMMARY

I have designed a protocol for the measurement of MPs based on light-scattering flow cytometry. MPs were identified based on size and expression of cell-specific antigens, and their absolute numbers quantified using a flow-based method. The limitation of my study is that MPs, by virtue of their small size, appear close to the machine noise in current flow cytometers. There is currently no standardised method for MP analysis, and it is likely that different pre-analytical variables and different methods of analysis influence the number of MPs detected. In view of this I ensured that all my patient samples were treated in an identical manner to minimise any *in vitro* effect on MP numbers and ensure that results from different patients were comparable.

CHAPTER IV

RESEARCH STUDY
METHODS AND RESULTS

4.1 INTRODUCTION

Patients with gynaecological malignancy have a significant risk of thrombosis, together with its associated morbidity and mortality. Further, patients with both a malignancy and a VTE event have a worse overall prognosis[19, 20, 47]. Thromboprophylaxis is known to reduce the incidence of VTE events, which in the perioperative period translates into a reduced mortality rate[6, 62-64]. However there is very little data on the use of primary thromboprophylaxis in patients with gynaecological malignancy outside the surgical setting. At present it is unclear what the optimal thromboprophylaxis regimen in this patient group is, especially as they also have a significant risk of bleeding. The risk factors for VTE are several. However we are currently unable to predict the likelihood of a VTE event in an individual. If patients at a higher risk of VTE events could be identified then primary thromboprophylaxis in such patients could potentially reduce the incidence of VTEs and improve the overall prognosis. Further studies to elucidate this are required before primary thromboprophylaxis can be advised for all patients with gynaecological malignancy.

MPs are thought to play an important role in haemostasis and thrombosis. They are also considered important in the patho-physiology of cancer cell survival, invasiveness and metastasis[99]. The number of circulating MPs, and MP-dependent function, have been variously reported to be increased in patients with underlying prothrombotic conditions, including malignancy. Patients with malignancy (with/without thrombosis) have also been found to have evidence of coagulation system activation[36, 78]. There are several published studies showing increased levels of markers of haemostatic activation such as D-dimers, TAT and PF1&2 in such patients[20, 142, 178].

It is therefore reasonable to assume that in patients with gynaecological malignancy the number of circulating MPs is likely to be increased, and that there will be evidence of coagulation system activation, with increased levels of D-dimers, TAT and PF1&2. I hypothesised that the number of circulating MPs in individual patients

with gynaecological malignancy may help identify patients at a significant risk of thrombosis, allowing primary thromboprophylaxis to be tailored accordingly. As far as I am able to ascertain there are no published studies looking at the number of MPs specifically in patients with gynaecological malignancy.

The aims and objectives of this research study are outlined in Chapter I, section 1.6, page 32. I have recruited patients presenting in the South East of Scotland Cancer Network (SCAN) with a confirmed diagnosis of gynaecological malignancy, and a control group of women with no underlying malignancy. I measured the number of circulating PMPs, LMPs, EMPs, TF+ve MPs and AV+ve MPs to establish whether MP levels are truly elevated in patients with gynaecological malignancy compared with women with no malignancy and to assess whether the number of circulating MPs can be used to establish the individual risk of thrombosis. I also measured D-dimers, TAT and PF1&2 in the two patient groups as indirect markers of haemostatic activation, and correlated their levels with the number of circulating MPs.

Statistical advice was obtained (Dr. Gillian Norrie BSc(Hons) Statistics) as to the sample size required in order to establish whether plasma MP levels could be used to assess the individual risk for VTEs. For MPs to be a useful predictor there would need to be a clinically meaningful difference in the mean MP numbers in patients with VTE compared to those with no VTE. The quoted incidence of VTE events in patients with gynaecological cancer is very variable: 9.8-57% in endometrial cancer[8], 0-34% in cervix cancer[9], 13.6-27% in ovarian cancer[7]. Based on these reports we predicted that approximately 25% of women with gynaecological malignancy would develop a VTE at some point during their investigation and treatment for cancer. From Chirinos et al[23] we hypothesised that the mean number of EMPs for patients with a VTE would be around 2000/μL, compared to approximately 400/μL for patients with no VTE. Assuming a common standard deviation of 2500/μL, then a consecutive series of around 140 patients with gynaecological malignancy would be expected to have around 35 women with a VTE event and 105 with no VTE. Such a study would have around 90% power to detect the hypothesised difference in mean EMP numbers between patients with a

VTE and patients with no VTE, at a 5% level of significance using a two sample t-test. Such a separation between patients with and without a VTE event may make EMP numbers a useful predictor of VTE. The study was also planned to recruit a similar number (n=140) of women without malignancy so that we could explore whether EMP numbers are a superior predictor of VTE in women with gynaecological cancer to those without.

4.2 Methods

4.2.1 Recruitment of malignant group and control group

All patients with gynaecological malignancy in SCAN are initially discussed and reviewed at the new patient gynae-oncology clinic at the Western General Hospital, Edinburgh. During 2009, patients attending this clinic with a confirmed diagnosis of gynaecological malignancy were invited to participate in this study (malignant group). Women attending the clinic were given written information (Letter of Invitation (Appendix A) and Patient Information Sheet for patients with gynaecological malignancy (Appendix B)) about this study after their initial consultation with the oncology team.

During the same period of time I also recruited a control group of women who were attending hospital for pre-operative clerking prior to proceeding with benign gynaecological surgery. This group of women (control group) did not have any underlying malignancy. Women attending the gynaecology pre-operative clerking clinic at the Royal Infirmary of Edinburgh were given written information (Letter of Invitation (Appendix A) and Patient Information Sheet for patients with benign gynaecological conditions requiring surgical treatment (Appendix C)) about this study at the time of their clinic appointment for pre-operative clerking.

All patients attending the above-mentioned clinics were approached about the study in a consecutive manner with no selection bias. A total of 69 patients with gynaecological malignancy and 45 patients with no underlying malignancy were invited to participate. Of these, 67 (97%) patients with gynaecological malignancy and 42 (93%) patients with no underlying malignancy agreed to participate (see Figure 4.1, page 79). The study was discussed with these patients in greater detail. They were given the opportunity to ask questions, and written informed consent was obtained from all patients enrolled in the study prior to any blood samples being taken (Consent Form, Appendix D1 & D2).

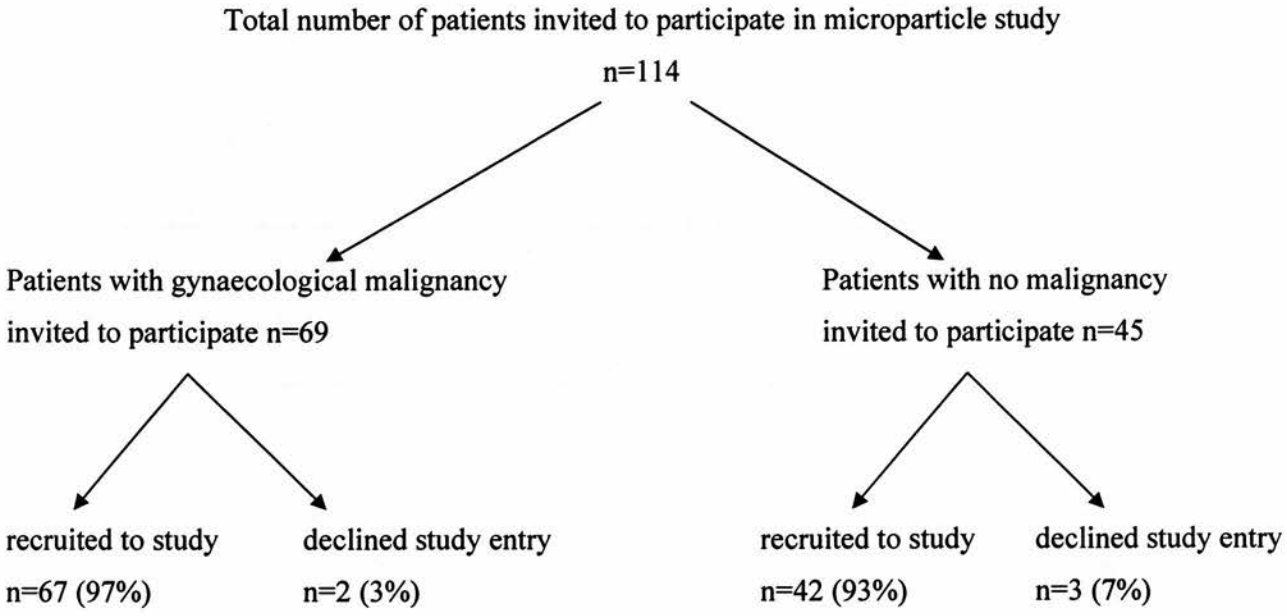


Figure 4.1: Recruitment tree

4.2.2. Blood sampling and handling

All patients with gynaecological malignancy recruited to the study (n=67) had a study blood sample taken as per study protocol at their first outpatient appointment with the oncology team.

In 7 of the above patients follow up blood samples were also taken at the end of their treatment to assess the effect of treatment on the number of circulating MPs. However this proved difficult to co-ordinate with the patients and was not continued. Six of these seven patients were treated with chemoradiotherapy or radiotherapy alone – in these patients the second blood sample was taken in their 5^{th} and final week of treatment just before their final treatment session. The 7^{th} patient was treated with chemotherapy only, and in this patient the second blood sample was taken in the 6^{th} and final week of treatment, just before the final chemotherapy session.

Of the patients with gynaecological malignancy who required surgery as part of their treatment, 7 provided a second sample of blood 24 hours after surgery. The rest declined a second blood sample post-operatively, mainly because they were not feeling very well.

Patients in the control group (n=42) had a blood sample taken as per study protocol at their pre-operative clerking appointment. In 18 of these patients a second blood sample was also taken 24 hours after surgery. The rest of the patients in the control group declined a second sample post-operatively, mainly because they were not feeling very well.

All samples were kept at room temperature pre-analysis and processed within 2 hours as per study protocol. Please refer to chapter III for full details.

4.2.3 Patient details

Patient details including histological diagnosis, disease stage, relevant past medical history, treatment given and follow up information was obtained by reviewing the patients' medical records. VTEs were defined as deep vein thrombosis and/or pulmonary embolism occurring up to one year prior to, or concurrent with, the diagnosis of malignancy, or during treatment and follow up of the malignancy in the malignant group, and within 3 months of surgery in the control group.

4.2.4 Ethics

The study was submitted to and approved by the Lothian Local Research Ethics Committee (REC reference number 08/S1103/51).

4.2.5 Statistics

Using the Shapiro-Wilk test, the parameters measured were found not to be normally distributed. Values are therefore expressed as the median and interquartile range (IQR). Comparisons between two independent groups of patients were carried out using the Mann Whitney *U* test, while comparisons between three or more independent groups were carried out using the Kruskal Wallis test. Multivariate analysis was carried out to allow for age adjustment.

The correlation between different variables was assessed using Spearman's nonparametric correlation coefficient (r). Paired samples were compared using the Wilcoxon Signed Ranks test. All reported probability values are two-tailed. Statistical significance was defined as a p value of < 0.05. Analyses were performed with the statistical package SPSS version 17.0 (SPSS Inc, Chicago, IL, USA).

4.3 Patient characteristics

4.3.1 Patient demographics

Of the women with a confirmed diagnosis of gynaecological malignancy presenting in SCAN during 2009, 67 agreed to take part in this study (malignant group). During the same period of time I also recruited a control group of 42 women who were attending hospital prior to proceeding with benign gynaecological surgery with no underlying malignancy. Table 4.1 is a summary of the demographics of the two groups of patients.

All the patients recruited in this study are women. The control group of women tended to be younger than the women in the malignant group (p=0.02), although not with a dissimilar age spread.

	Malignant group	**Control group**
n	67	42
Age median (range)	65 yrs (26-89yrs)	57 yrs (38-83yrs)
Diagnosis n (%)	Ovarian cancer n=24 (36%) Endometrial cancer n=24 (36%) Cervical cancer n=13 (19%) Vulval cancer n=5 (7.5%) Vaginal cancer n=1 (1.5%)	Prolapse n=18 (43%) Benign fibroids n=18 (43%) Benign cysts n=6 (14%)

Table 4.1: Demographics of the two patient groups.

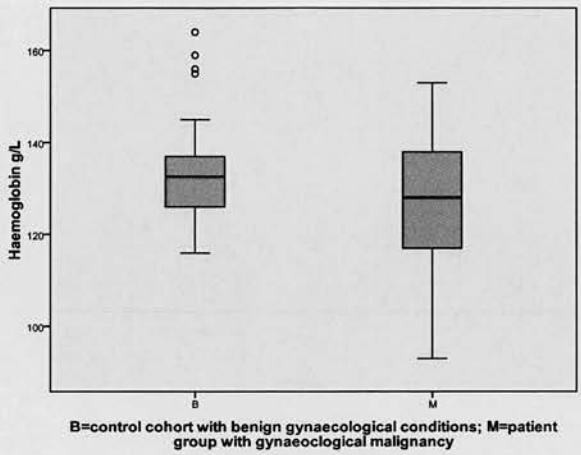
4.3.2 Haematological and Renal Parameters

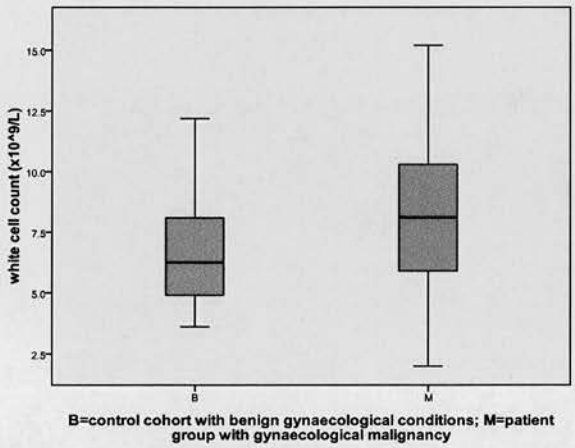
The haematological parameters (FBC) and renal function (EGFR) of the two patient groups at the time of their recruitment to my study are summarised in Table 4.2, page 84. Using the Shapiro-Wilk test the FBC results were found not to be normally distributed – Figure 4.2, page 85.

The malignant group tended to have a lower haemoglobin level, however this difference did not reach statistical significance. The white blood count (wbc) was higher in the malignant group, reaching statistical significance ($p=0.001$), while there was no overall difference in the platelet count between the two groups. Patients with gynaecological malignancy were more likely to have impaired renal function (EGFR <60mls/min).

	Malignant group (M) (n=65)	***Control cohort (B) (n=42)***	**p value (M *vs* B)**
Hb (g/L) median (IQR)	128 (115.5 – 138)	*132.5 (126 - 137)*	0.083
wbc ($x10^9$/L) median (IQR)	8.1 (5.9 – 10.4)	*6.25 (4.9 - 8.1)*	0.001
Platelets ($x10^9$/L) median (IQR)	282 (229.5 – 381.5)	*263.5 (225.3 - 314.8)*	0.141
No. of pts with normal EGFR (%) (>60 mls/min)	54 (83%)	*40 (95%)*	n/a
No. of pts with reduced EGFR (%) (<60 mls/min)	11 (17%) (range 27-57 mls/min)	*2 (5%) (52 & 57 mls/min)*	n/a

Table 4.2: Summary of the haematological and renal parameters. Two of the patients with gynaecological malignancy did not have a full blood count and renal function checked at recruitment to the study (n=65 instead of 67). M refers to the patients with gynaecological Malignancy, B refers to the control group with Benign gynaecological conditions. p value result for 2-tailed significance testing using the Mann Whitney *U* test for 2 independent samples. Haemoglobin (Hb), white blood count (wbc), estimated glomerular filtration rate (EGFR), patients (pts), number (no.).





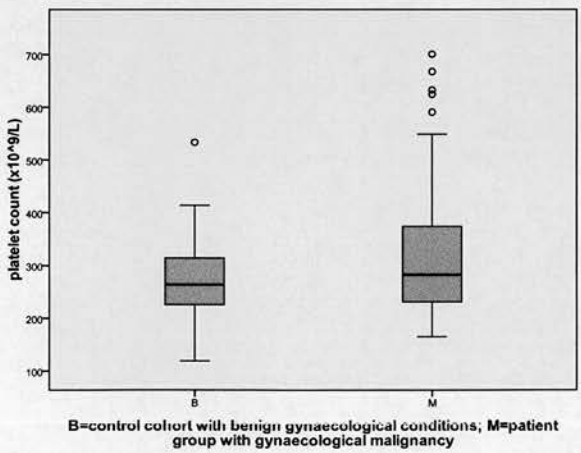


Figure 4.2: Box plots showing the distribution of the haematological parameters.

4.3.3 Treatment

The women in the malignant group were treated with a variety of different protocols, depending on their specific malignancy, disease stage and co-morbidities. The surgical treatment of the control group also varied according to the specific condition requiring surgery. Table 4.3 is a summary of the treatment received by the patients in this study.

	Diagnosis	**Treatment**
Malignant group (n=67)	Cervical cancer (n=13)	TAH BSO (1) TAH BSO + Cisplatin + radiotherapy (3) Cisplatin + radiotherapy (8) Palliative radiotherapy (1)
	Endometrial cancer (n=24)	TAH BSO (4) TAH BSO + radiotherapy (14) TAH BSO + Carboplatin & Paclitaxel + radiotherapy (3) TAH BSO + Carboplatin & Paclitaxel (1) Carboplatin (2)
	Vulval cancer (n=5)	Surgical excision (2) Surgical excision + Cisplatin + radiotherapy (1) Palliative radiotherapy (2)
	Ovarian cancer (n=24)	TAH BSO + Carboplatin & Paclitaxel (10) TAH BSO (6) Carboplatin & Paclitaxel (6) Hormone treatment (1) Unfit for treatment (1)
	Vaginal cancer (n=1)	Radiotherapy (1)
Control Group (n=42)	Benign fibroids (n=18)	Myomectomy (3) TAH BSO (15)
	Pelvic floor prolapse (n=18)	Pelvic floor repair (10) TAH BSO + pelvic floor repair (8)
	Benign ovarian cysts (n=6)	BSO (3) TAH BSO (3)

Table 4.3: Summary of the treatment delivered to the patients in the study.
Total abdominal hysterectomy (TAH), bilateral salpingo-oophorectomy (BSO)

4.3.4 Incidence of Venous Thromboembolism

Any patients presenting with symptoms suggestive of venous thromboembolic events were objectively and appropriately investigated by imaging techniques according to their symptoms (CT scan and/or lower limb dopplers). Imaging (CT scan) was also carried out on all women with malignancy to stage the cancer and assess response to treatment. Routine imaging was not carried out in the control group, although patients in the control group were investigated appropriately if they developed symptoms suggestive of VTE.

Of the 67 women in the malignant group, 5 were diagnosed with a VTE episode – 4 (2 ovarian carcinoma, 2 vulval carcinoma) at the time their malignancy was first diagnosed on a staging scan, and one patient (ovarian carcinoma) while on chemotherapy (Carboplatin and Paclitaxel), after investigation of symptoms suggestive of a pulmonary embolism. None of the patients requiring surgical treatment of their malignancy were diagnosed with a thrombotic event in the perioperative period. Likewise, no patients receiving radiotherapy were diagnosed with a thrombotic event. There were no arterial events.

Of the 4 patients diagnosed with a VTE event concurrent with their diagnosis of malignancy, 1 had a coincidental diagnosis of both lower limb deep vein thrombosis and pulmonary embolism on staging CT scan and 2 had a coincidental diagnosis of only pulmonary embolism on staging CT scan. The 4^{th} patient developed symptoms suggestive of a pulmonary embolism a few days after her diagnosis of cancer (and prior to commencing treatment) – the diagnosis of pulmonary embolism was confirmed on CT pulmonary angiogram. The patient with a VTE event diagnosed while on chemotherapy had a pulmonary embolus confirmed with CT pulmonary angiography after developing symptoms suggestive of such a diagnosis.

All 4 patients who presented with a VTE at the same time their malignancy was diagnosed had extensive disease and were only fit for palliative treatment, either by virtue of the extent of their disease or because of general frailty. Two of these

patients (40% of the patients with a VTE) passed away very quickly. Please refer to Table 4.4, page 89 for a summary of the patients who developed VTE events.

None of the women in the control group were diagnosed with a thrombotic event within 3 months of surgery. All surgical patients (both in the malignant group and in the control group) were routinely managed with primary thromboprophylaxis with low molecular weight heparin and TED stockings in the perioperative period.

4.3.5 Mortality rate

Of all the patients in this study, 4 (6%) of the malignant group (3 ovarian carcinoma and 1 vulval carcinoma) died during the follow up period (median follow up of 11 months; range 5-17 months). Two of these patients had been diagnosed with venous thrombosis at presentation with gynaecological malignancy, so that 40% (2 out of 5 patients with a VTE event) of the patients with a VTE event died, compared to 3.2% (2 out of 62 patients with no thrombosis) of the patients with no thrombosis. None of the control group died during the same observation period.

Diagnosis (n)	No. of pts with VTE diagnosed concurrent with ca (%)	No. of pts with VTE diagnosed at a later date (%)	Total no. of pts with VTE events (%)	No. of pts with CVA or MI
Ovarian ca (24)	2 (8.3%)	1 (4.2%)	3 (12.5%)	0
Vulval ca (5)	2 (40%)	0	2 (40%)	0
Cervical ca (13)	0	0	0	0
Endometrial ca (24)	0	0	0	0
Vaginal ca (1)	0	0	0	0
Total malignant group (67)	4 (5.97%)	1(1.5%)	5 (7.5%)	0
Control group (42)	*0*	*0*	*0*	*0*

Table 4.4: Summary of the thrombotic events in the study patients. VTE (venous thromboembolism) refers to deep vein thrombosis (DVT) and/or pulmonary embolism (PE). Number (no.), patients (pts), cancer (ca), cerebrovascular accident (CVA), myocardial infarction (MI).

4.4 Study Results

4.4.1 Distribution of the measured parameters

Using the Shapiro-Wilk test, the number of circulating MPs and the level of D-dimers, TAT and PF1&2 were found not to be normally distributed – please refer to Figure 4.3, page 90.

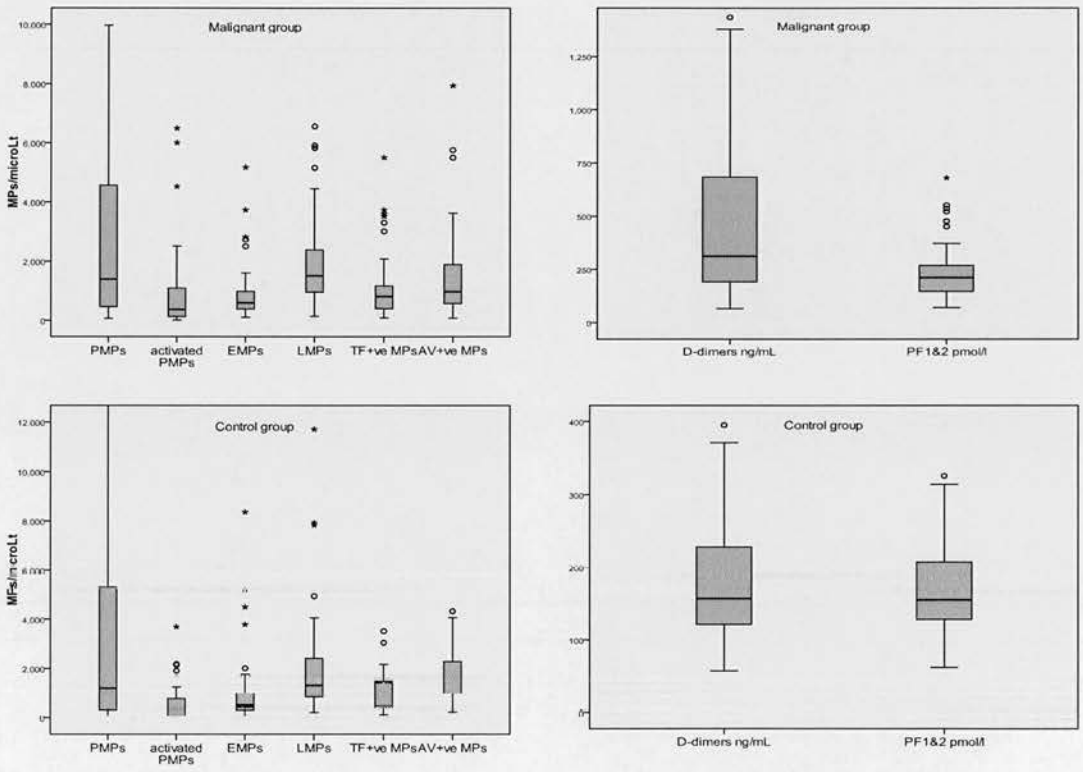


Figure 4.3: Box plots showing the distribution of the number of microparticles (MPs) and the level of D-dimers and PF1&2 for the malignant group and the control group. The thrombin-antithrombin results have not been included due to the fact that a very different scale is required to show these results. They were also not normally distributed.

4.4.2 MP results

The MP results are summarised in Table 4.5 and Figure 4.4, page 92. The number of circulating MPs (PMPs, LMPs, EMPs, AV+ve MPs) was very similar between the two groups with no statistically significant difference. There was also no difference in the number of circulating TF+ve MPs between the two groups. This was also the case when the MP levels were analysed according to tumour stage, specific cancer diagnosis (i.e. cervical, endometrial, ovarian, vulval and vaginal cancer) and specific reason for requiring benign gynaecological surgery (i.e. pelvic floor prolapse, benign ovarian cysts, benign fibroids) – please refer to Figure 4.5, page 93.

MP subtype	***Control group***		**Malignant group**		**p value**
	Median (/µL plasma)	***IQR (/µL plasma)***	**Median (/µL plasma)**	**IQR (/µL plasma)**	
PMPs (anti-CD41)	*1195*	*296-5351*	1396	397-4652	0.35
activated PMPs (anti-CD41 & anti-CD62P)	*336*	*104-782*	370	126-1091	0.3
EMPs (anti-CD144)	*523*	*299-995*	589	381-985	0.476
LMPs (anti-CD45)	*1315*	*844-2436*	1510	950-2409	0.641
TF+ve MPs (anti-CD142)	*981*	*478-1486*	805	386-1169	0.214
AV+ve MPs	*1609*	*998-2289*	972	567-1938	0.051

Table 4.5: Number of microparticles (MPs) in the two patient groups according to the specific cell of origin, tissue factor (TF) positivity and annexin V (AV) positivity. There was no statistically significant difference. p value result for 2-tailed significance testing using the Mann Whitney *U* test for 2 independent samples. Interquartile Range (IQR), platelet-derived MPs (PMPs), endothelial-cell derived MPs (EMPs), leucocyte-derived MPs (LMPs).

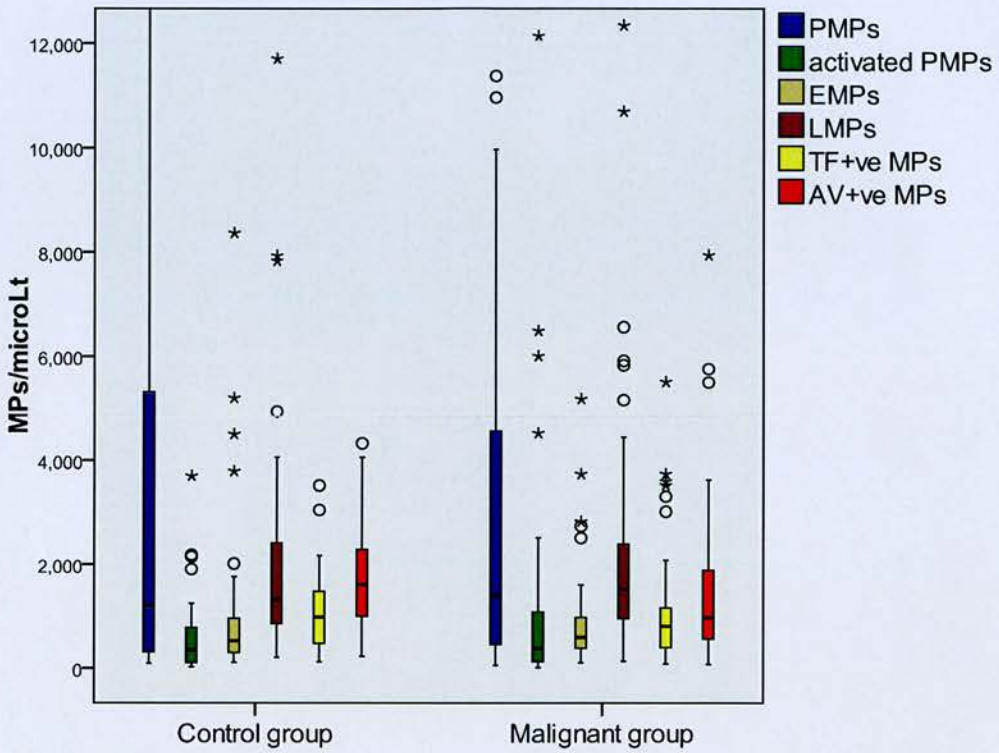


Figure 4.4: Box plot of the microparticle (MP) results. There is no statistically significant difference in the number of circulating MPs between the two groups.

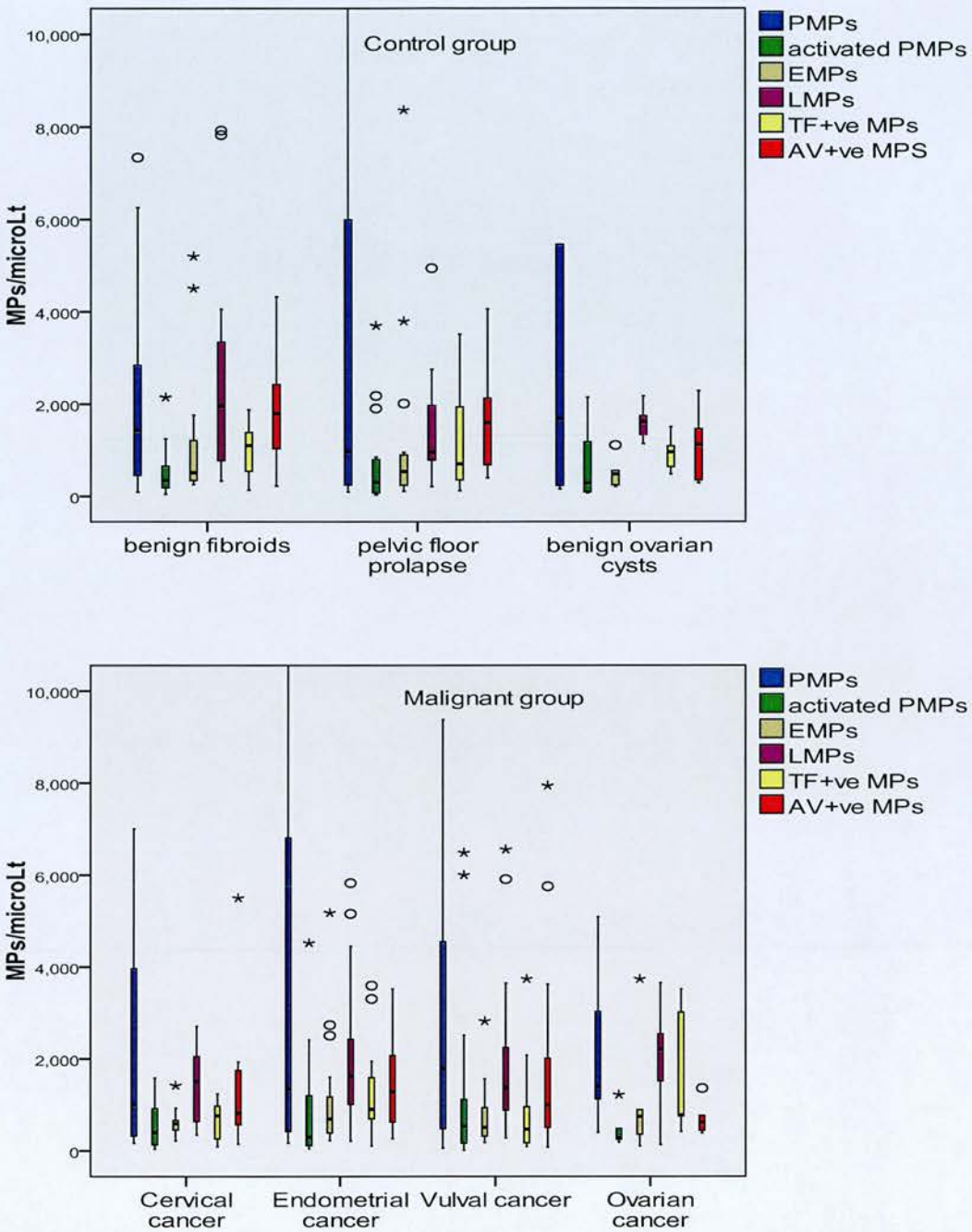


Figure 4.5: Box plots of the microparticle (MP) results by specific diagnosis. The results of the patient with vaginal cancer are not shown above due to the fact that there was only one patient with this diagnosis. There was no statistically significant difference in the number of circulating MPs between the different groups. p value ranging from 0.217 to 0.986, Kruskal Wallis test.

There was a weak to moderate correlation amongst the different subtypes of circulating MPs. Patients with increased levels of PMPs also had increased levels of EMPs and LMPs. This was true for both the malignant patients and the control group. Table 4.6 is a summary of these correlations.

Spearman's Correlation		
MP subtypes	**p value**	**Correlation coefficient r**
PMPs & activated PMPs	**<0.001**	0.96
PMPs & EMPs	**<0.001**	0.404
activated PMPs & EMPs	**<0.001**	0.398
PMPs & LMPs	**<0.001**	0.342
activated PMPs & LMPs	**<0.001**	0.339
EMPs & LMPs	**<0.001**	0.478

Table 4.6: Summary of the Spearman's correlation between the different cell specific microparticle (MP) subtypes.

4.4.3 Relative proportions of MP cell-specific subtypes

PMPs (anti-CD41) and LMPs (anti-CD45) together made up the majority of measured MPs, with EMPs (anti-CD144) making up a smaller percentage – please refer to Table 4.7.

MP subtype	**Malignant group (% of PMPs+EMPs+LMPs)**	***Control group (% of PMPs+EMPs+LMPs)***
PMPs	44.1	*38.38*
LMPs	38.72	*41.86*
EMPs	17.18	*19.75*

Table 4.7: The relative proportions of circulating microparticles (MPs) in the two patient groups. The above results refer to the median percentage the different MP subtypes make up when compared to the total number of cell specific MPs measured. For example, PMPs are expressed as a percentage of PMPs+EMPs+LMPs. Platelet-derived MPs (PMPs), endothelial-cell derived MPs (EMPs), leucocyte-derived MPs (LMPs).

4.4.4 AV+ve MPs

As mentioned in chapter III I measured the total number of AV+ve MPs, but was unable to identify their cell of origin. The total number of AV+ve MPs was in the majority of patients significantly less than the total number of MPs detected by the cell-specific antigens (i.e. PMPs+EMPs+LMPs) in that patient. This means that a significant number of the MPs measured were AV-ve (Figure 4.6). In a small number of patients the total number of circulating AV+ve MPs was more than the total number of PMPs, EMPs and LMPs measured together. In the latter patients some of the AV+ve MPs must have been released by other cell types such as red cells and tumour cells.

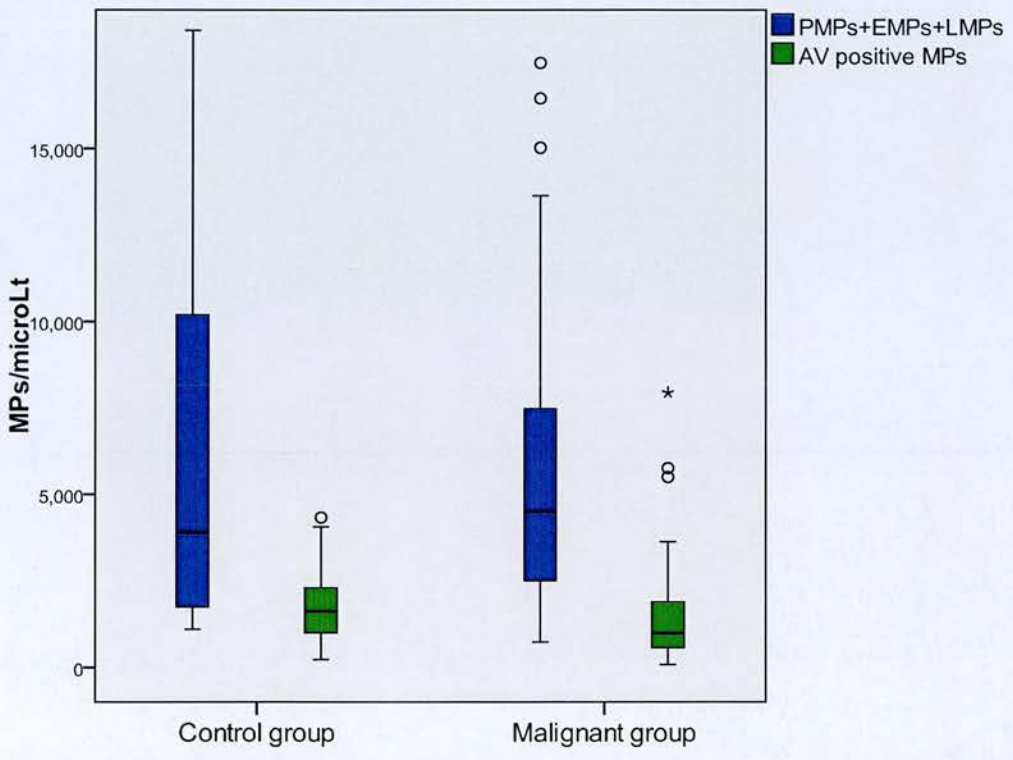


Figure 4.6: Bar chart showing the total number of AV+ve MPs in relation to the total number of cell specific MPs identified i.e. PMPs+EMPs+LMPs. AV+ve MPs make up a minority of all microparticles detected. Annexin V (AV), platelet-derived MPs (PMPs), endothelial-cell derived MPs (EMPs), leucocyte-derived MPs (LMPs).

4.4.5 Correlation of MPs and age

Using Spearman's rank testing, there was no correlation between the number of circulating MPs and the patients' age for both patient groups – Figure 4.7, page 98.

4.4.6 Correlation of MPs with haematological and renal parameters

There was a weak correlation ($p=0.005$, $r=0.269$) between the patients' peripheral blood platelet count and the number of circulating PMPs. However there was no correlation between the level of haemoglobin, white cell count or EGFR and the number of circulating MPs (Figure 4.8, page 99-100).

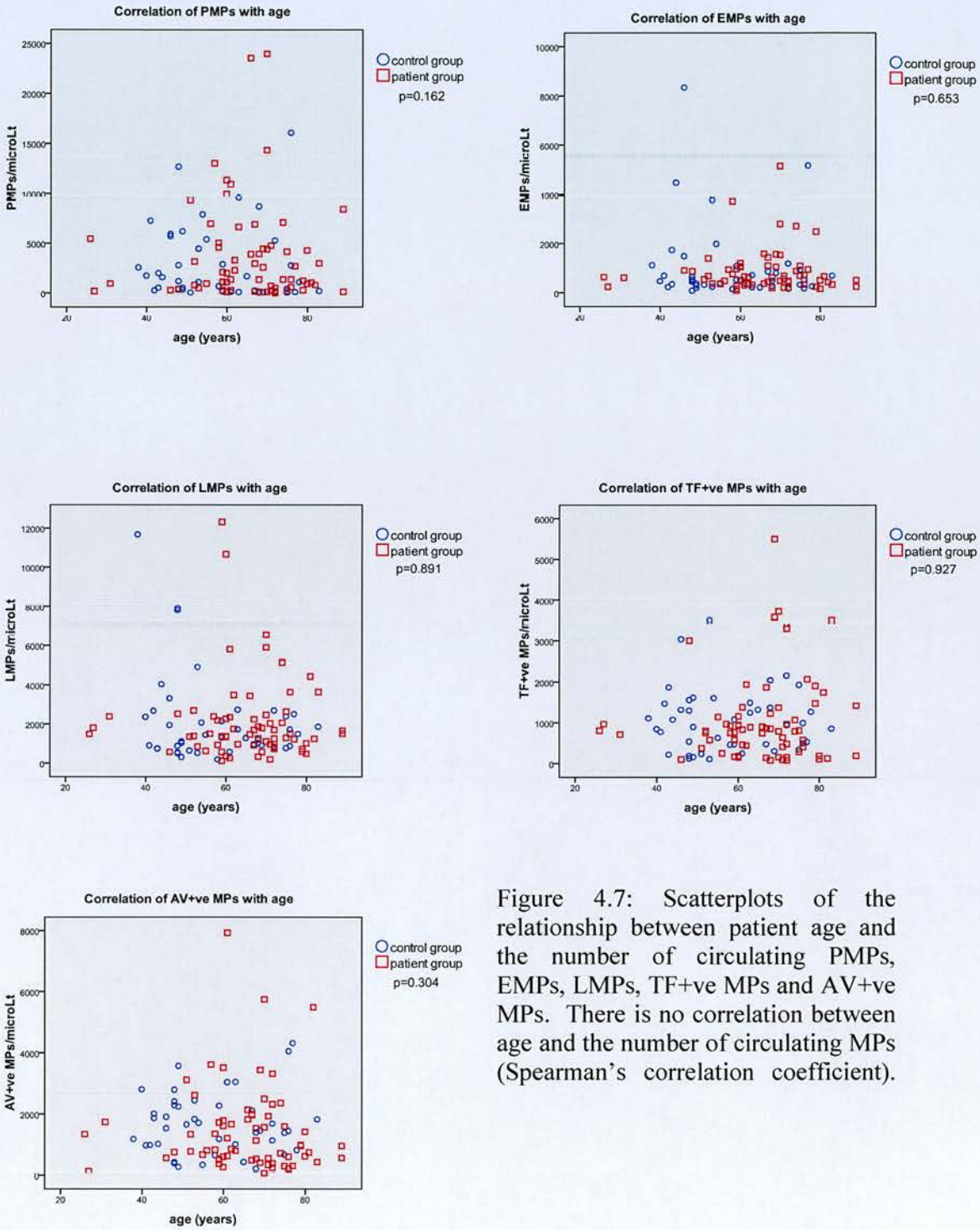


Figure 4.7: Scatterplots of the relationship between patient age and the number of circulating PMPs, EMPs, LMPs, TF+ve MPs and AV+ve MPs. There is no correlation between age and the number of circulating MPs (Spearman's correlation coefficient).

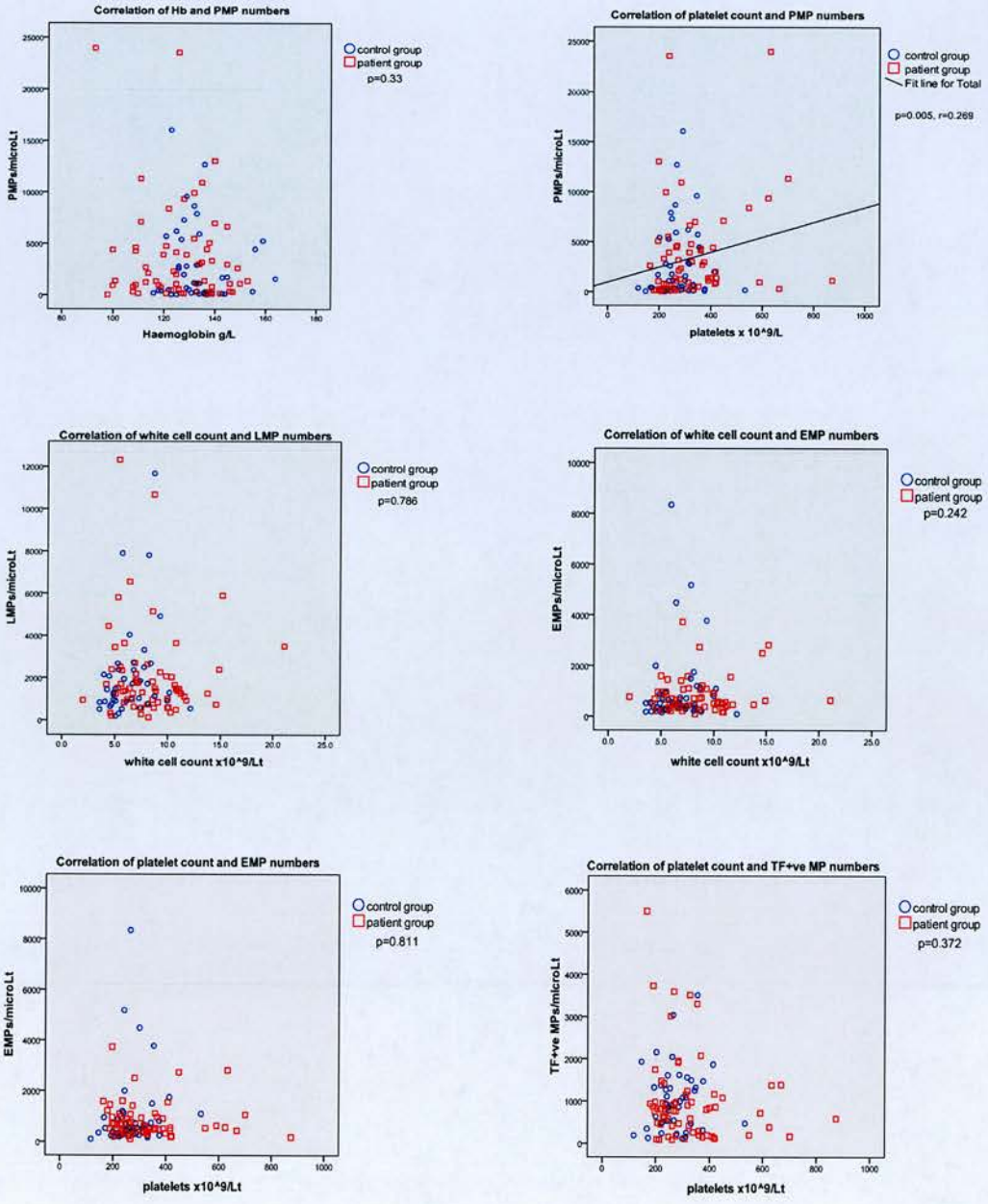


Figure 4.8a: Scatterplots of haematological and renal parameters and the number of circulating microparticles (MPs) (Spearman's correlation coefficient). There was a weak correlation between the peripheral blood platelet count and the number of circulating platelet-derived MPs (PMPs).

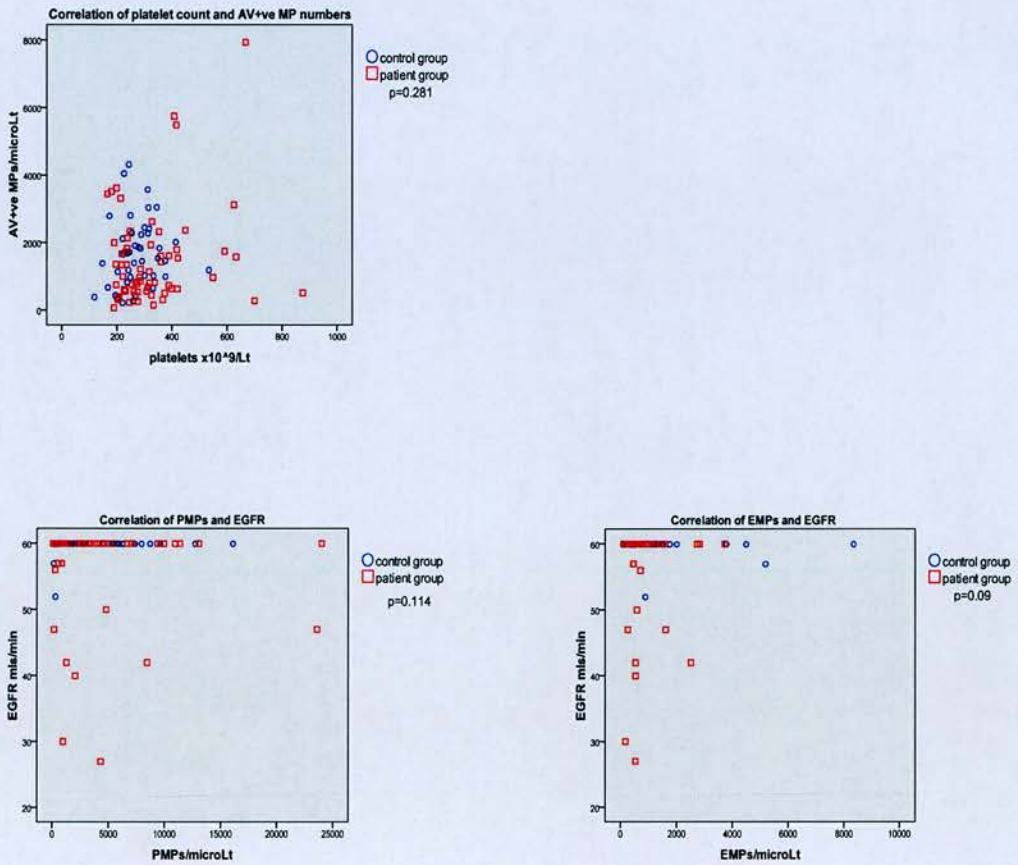


Figure 4.8b: Scatterplots of haematological and renal parameters and the number of circulating microparticles (MPs) (Spearman's correlation coefficient). There was a weak correlation between the peripheral blood platelet count and the number of circulating platelet-derived MPs (PMPs).

4.4.7 Residual platelet count in PFP

There was no correlation between the patients' peripheral blood platelet count and the residual platelet count in the PFP (p=0.55), and no significant difference in the residual platelet count in the PFP between the two patient groups (p=0.707). There was however a statistically significant strong correlation between the residual platelet count in the PFP and the number of PMPs detected by flow cytometry. This suggests that the residual contaminating platelets (although at a low level in all samples) were interfering with the PMP detection probably due to the presence of small platelets being detected in the 1 μm gate – Figure 4.9.

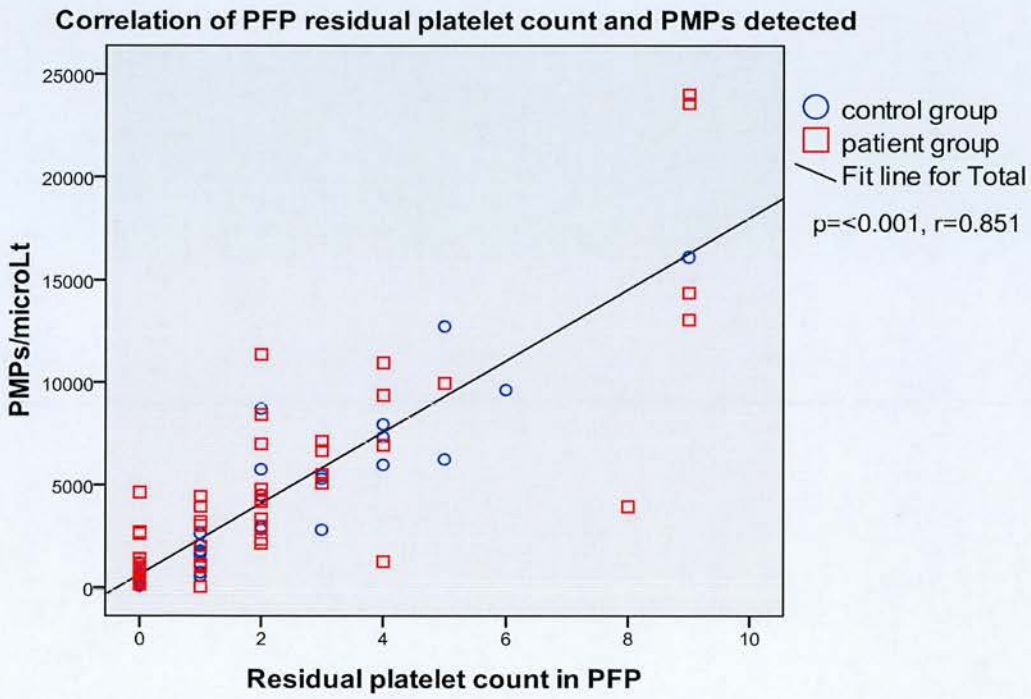


Figure 4.9: Scatterplot showing the statistically significant strong correlation between the residual platelet count ($x10^9$/L) in the platelet free plasma (PFP) and the number of platelet-derived microparticles (PMPs) measured (Spearman's correlation coefficient).

4.4.8 MPs and thrombosis

Five women from the malignant group and none from the control group were diagnosed with a VTE during the follow up period (median follow up of 11 months, range 5-17 months) – Table 4.4, page 89. There was no statistically significant difference in the number of MPs between patients with a VTE and patients with no VTE; however the numbers are too small to draw any final conclusion. The results are summarised in Table 4.8.

MP subtype	*Patients with VTE n=5*		**Patients with no VTE n=104**		p value
	Median (/µL plasma)	***IQR (/µL plasma)***	**Median (/µL plasma)**	**IQR (/µL plasma)**	
PMPs (anti-CD41)	*3025*	*1770-14227*	1249	326-4762	0.14
activated PMPs (anti-CD41 & anti-CD62P)	*506*	*364-3361*	354	109-1060	0.265
EMPs (anti-CD144)	*746*	*325-2193*	546	354-967	0.505
LMPs (anti-CD45)	*1517*	*853-4784*	1436	893-2402	0.602
TF+ve MPs (anti-CD142)	*417*	*172-2450*	850	456-1332	0.633
AV+ve MPs	*615*	*492-3665*	1357	649-2104	0.572

Table 4.8: Comparison of the number of microparticles (MPs) in patients with venous thromboembolism (VTE) and patients with no VTE. There was no statistically significant difference. p value for 2-tailed significance testing was calculated using the Mann Whitney *U* test for 2 independent samples. Interquartile Range (IQR), platelet-derived MPs (PMPs), endothelial-cell derived MPs (EMPs), leucocyte-derived MPs (LMPs), tissue factor (TF), annexin V (AV).

4.4.9 MPs and mortality

Four of the women in the malignant group and none of the patients in the control group died during the observation period (median follow up of 11 months, range 5-17 months). There was no statistically significant difference in the number of circulating MPs in the patients who died (p value ranging from 0.204 to 0.895 for the different MP subtypes, Mann Whitney *U* test), although the numbers are too small to draw any firm conclusions.

4.4.10 Effect of chemo/radiotherapy on MP levels

Seven of the women with gynaecological malignancy provided a second blood sample on their last day of chemotherapy and/or radiotherapy treatment. The demographics of these 7 patients are summarised in Table 4.9.

There was no statistically significant difference between the number of MPs measured prior to starting treatment and the number of MPs at the end of treatment (p value ranging from 0.091 to 1.0 for the different MP subtypes, Wilcoxon Signed Ranks test). However there was an overall tendency for the number of circulating PMPs, EMPs and LMPs detected at the end of treatment to be present in smaller numbers than prior to starting treatment. At the same time these patients had a lower number of platelets and white cells in their peripheral blood at the end of treatment compared to their peripheral blood results at the time their first sample for MP analysis was taken pre-treatment. This suggests that the trend in the number of circulating MPs, particularly the PMPs and LMPs, may partly reflect the number of circulating platelets and white cells in their peripheral blood respectively. The results for TF+ve MPs and AV+ve MPs are more variable – Figure 4.10, page 105.

Patient	Diagnosis	Treatment given
1	Cervical ca	Cisplatin and radiotherapy
2	Endometrial ca	Radiotherapy
3	Endometrial ca	Radiotherapy
4	Cervical ca	Cisplatin and radiotherapy
5	Ovarian ca	Carboplatin and Paclitaxel
6	Cervical ca	Cisplatin and radiotherapy
7	Cervical ca	Cisplatin and radiotherapy

Table 4.9: Demographics of patients with paired blood samples, one sample taken prior to starting treatment and another sample taken at the end of treatment. Cancer (ca)

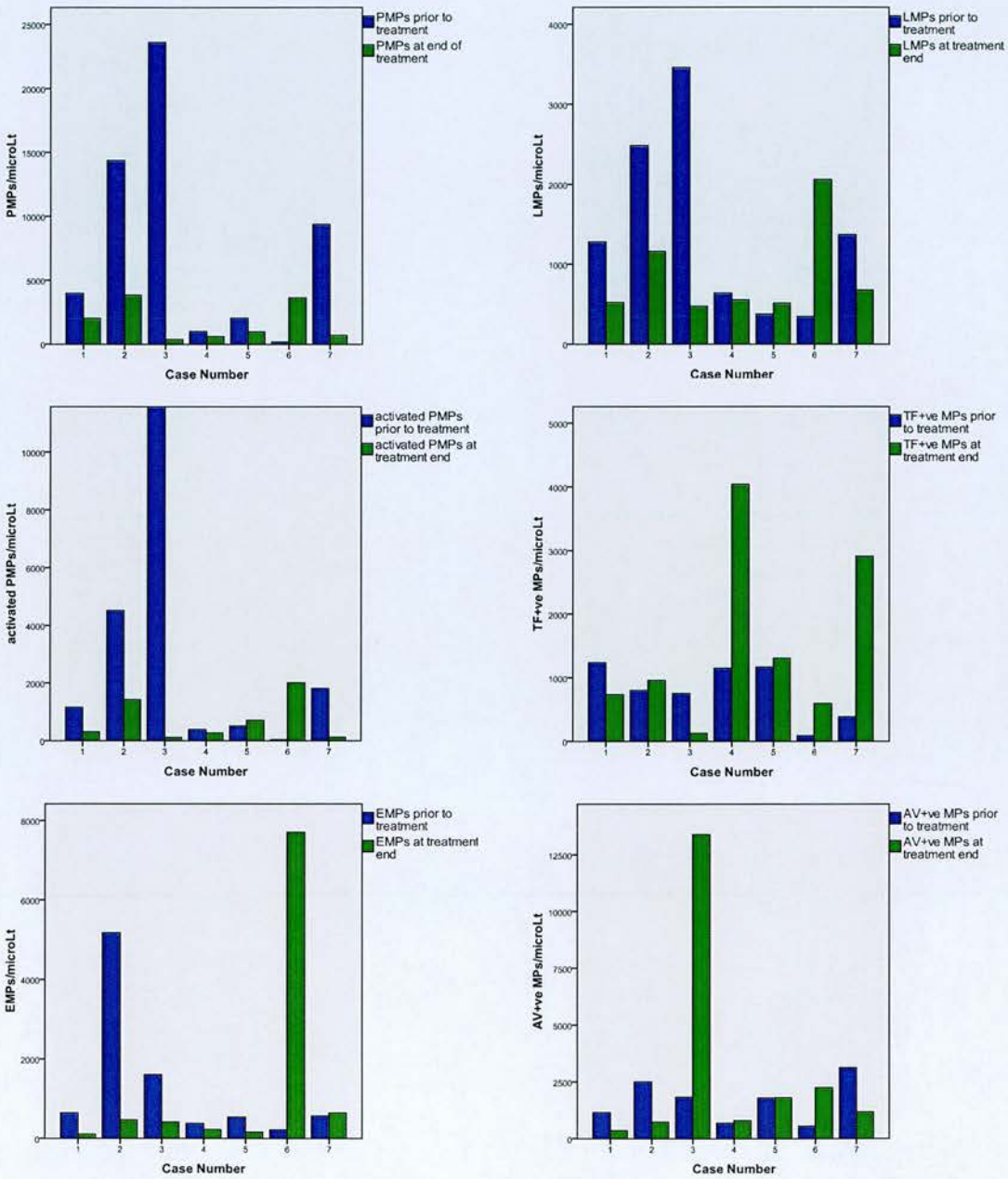


Figure 4.10: Bar charts of the number of microparticles (MPs) detected at the start of cancer treatment and at the end of treatment. There was no statistically significant difference between the number of MPs measured prior to starting treatment and the number of MPs at the end of treatment (p=0.091-1.0, Wilcoxon Signed Ranks test). There was however a trend for the number of circulating MPs detected at the end of treatment to be fewer than prior to starting treatment.

4.4.11 Postoperative levels of MPs

In a number of patients with gynaecological malignancy, treatment included surgery. Seven of these patients provided a second blood sample for MP analysis 24 hours postoperatively, to assess the effect of surgery on the number of MPs. I also obtained a second blood sample 24 hours postoperatively from 18 of the women in the control group.

The number of MPs detected postoperatively compared to the number of MPs preoperatively was very variable with no definite trend - in some patients the number of MPs postoperatively was significantly higher than that preoperatively and vice versa. Overall there was no statistically significant difference between the number of circulating MPs preoperatively and postoperatively (p value ranging from 0.176 to 0.612 for the different MP subtypes, Wilcoxon Signed Ranks test) – please refer to Figure 4.11, page 108 and 109.

4.4.12 Decision to stop study recruitment

As per statistical advice from Dr. Gillian Norrie, BSc(Hons) Statistics, the original plan was to recruit 140 patients each in the malignant group and the control group in order to have a 90% probability of detecting a difference in MP numbers between patients with a VTE event and patients with no VTE (see page 76).

Interim statistical analysis was carried out after 12 months of recruiting patients (n=67 patients with gynaecological malignancy; n=42 controls). There was no statistically significant difference in the number of MPs measured between the malignant group and control group for all subtypes of MPs. The number of patients diagnosed with a VTE was too small to assess the relationship between VTEs and MPs in any formal statistical analysis. As the assumptions made for the power calculations were shown to be incorrect for this study population, it was unlikely that we would be able to identify a difference in MP numbers between patients with a VTE and patients with no VTE. It was also unlikely that a statistically significant difference in MP numbers between cancer patients and controls would be demonstrable. In view of this a decision was made to stop recruiting patients. It was

decided to collect data on the incidence of VTEs in the overall population of patients diagnosed with gynaecological cancer in SCAN (the geographical area from where the study patients were recruited) to establish whether the patients recruited to this study were representative of the overall SCAN patients with gynaecological cancer or not.

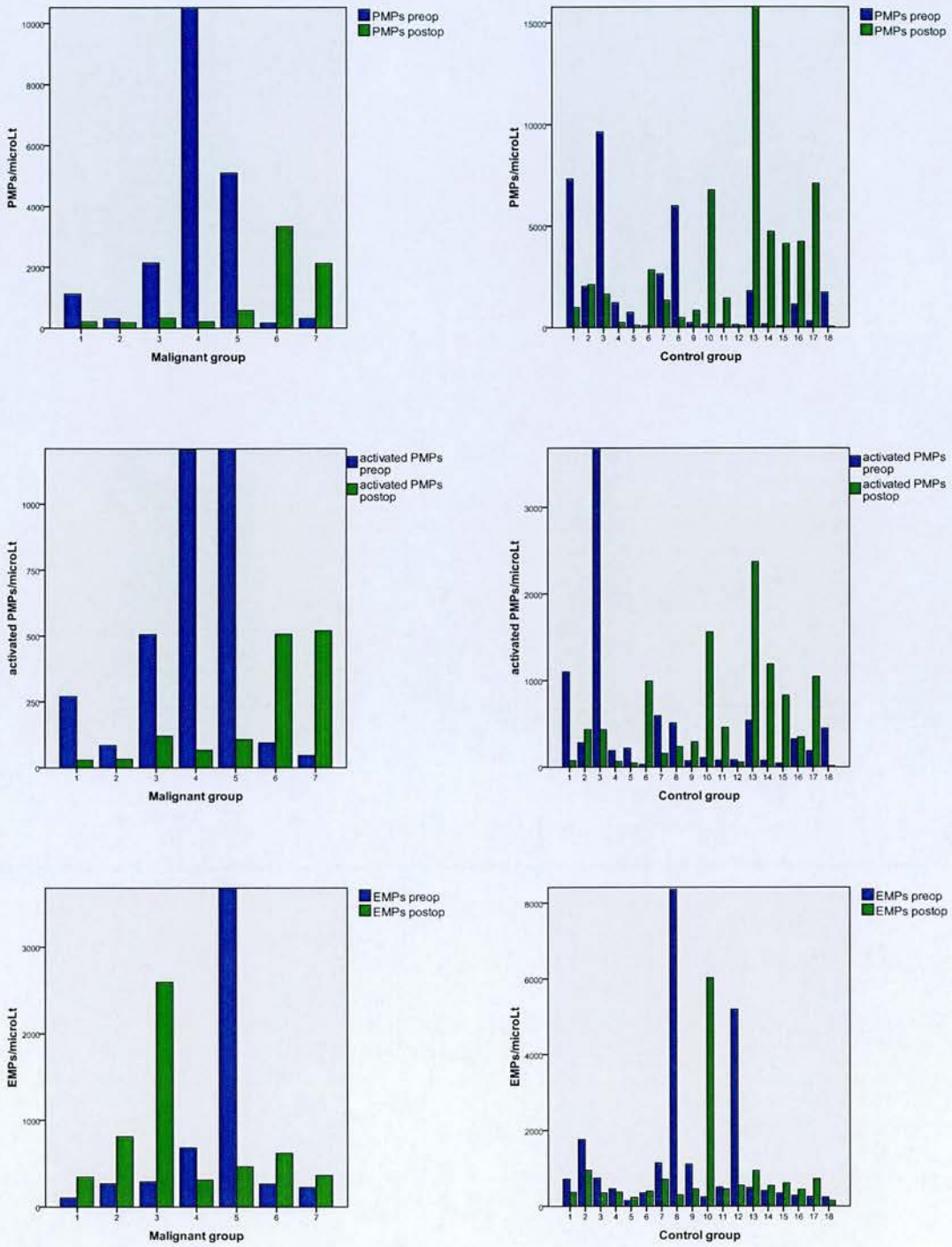


Figure 4.11a: Comparison of the numbers of circulating microparticles (MPs) preoperatively and postoperatively in both patient groups. The number of MPs detected postoperatively compared to the number of MPs preoperatively was very variable with no definite trend.

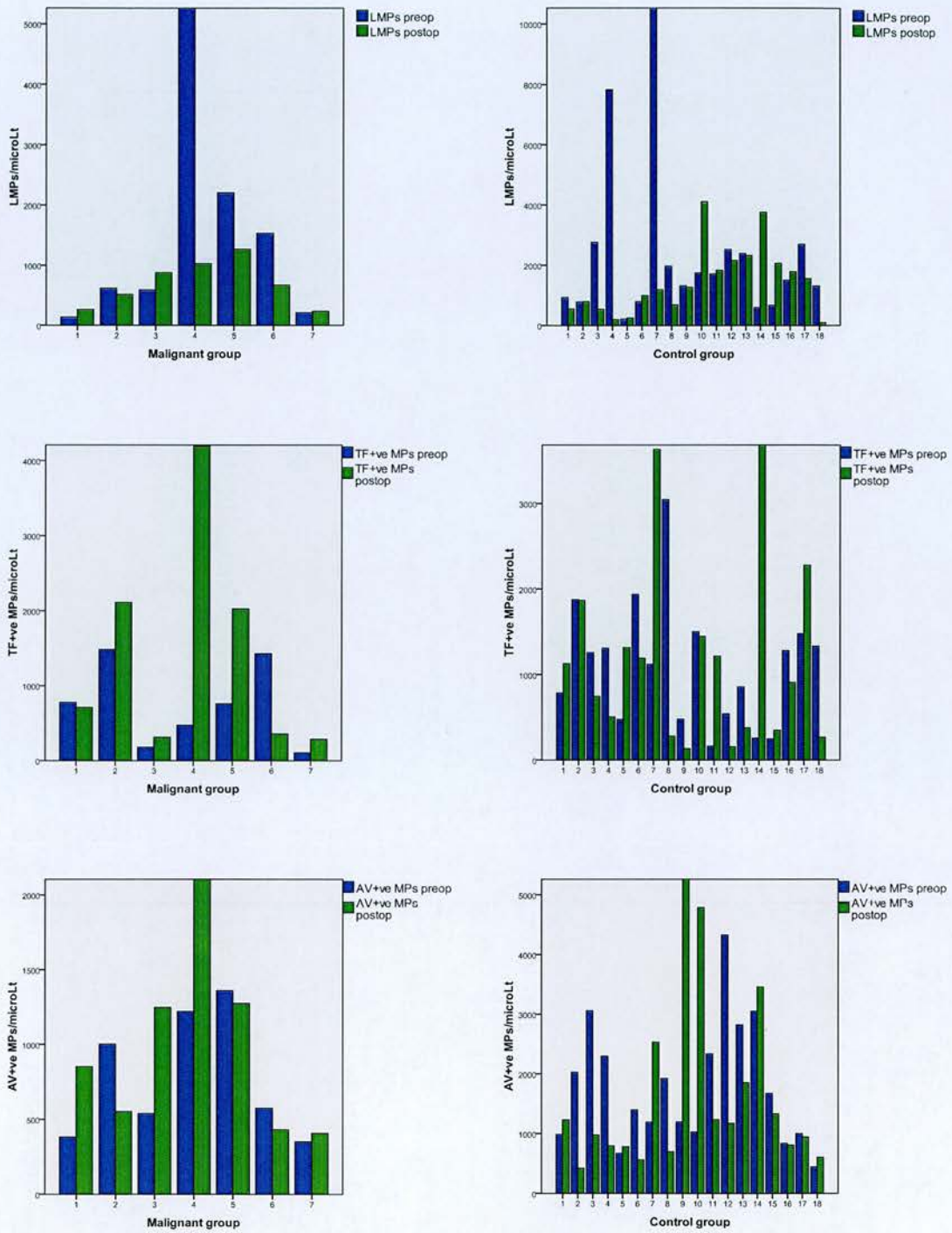


Figure 4.11b: Comparison of the numbers of circulating microparticles (MPs) preoperatively and postoperatively in both patient groups. The number of MPs detected postoperatively compared to the number of MPs preoperatively was very variable with no definite trend.

4.4.13 D-dimers, TAT and PF1&2 Results

The results for the markers of haemostatic activation are summarised in Table 4.10. There was a statistically significant increased level of D-dimers ($p<0.001$) and PF1&2 ($p=0.009$) in patients with gynaecological malignancy, evidence of haemostatic activation in this patient group – Figure 4.12, page 111.

	Control group *n=42* *Median (IQR)*	**Malignant group** n=67 Median (IQR)	**Mann Whitney *U* test** p=
D-dimers (<230 ng/mL)	*156.5* *(120.75-235.5)*	312 (190-688)	**<0.001**
TAT (1-4.1 µg/L)	*3.45* *(2.4-4.25)*	3.7 (3-6.1)	0.087
PF1&2 (69-229 pmol/L)	*155* *(125-212)*	211 (146-270)	**0.009**

Table 4.10: Summary of the D-dimer, TAT and PF1&2 results for the two patient groups. The D-dimer and PF1&2 results were increased in the malignant group, reaching statistical significance. Interquartile range (IQR)

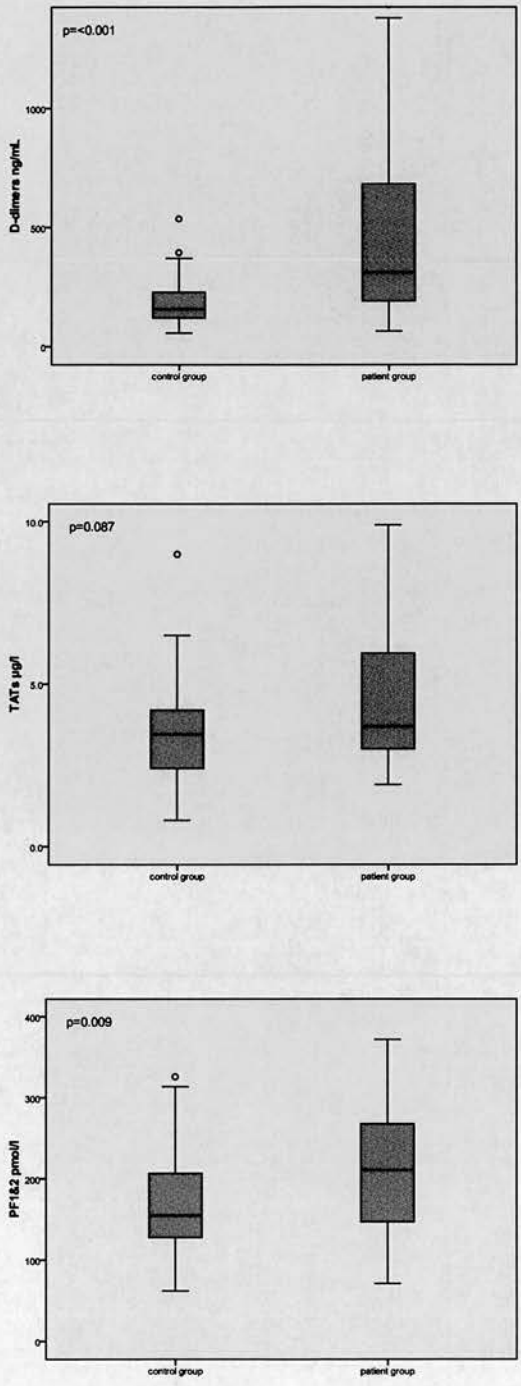


Figure 4.12: D-dimer, TAT and PF1&2 levels in patients with gynaecological malignancy compared to the control group. There was a statistically significant increased level of D-dimers and PF1&2 in the patients with malignancy.

4.4.14 Correlation of markers of haemostatic activation and age

There was a moderate, statistically significant correlation between D-dimer levels and age and between PF1&2 levels and age. There was no correlation between the level of TAT and age – Figure 4.13, page 113.

In view of the correlation between D-dimer and PF1&2 levels and age, multivariant analysis was carried out to establish whether there was a statistically significant difference in the level of these markers of haemostatic activation between patients with gynaecological malignancy and the control group once the results were adjusted for age. The results are presented in Figure 4.14, page 114. D-dimer levels ($p=0.004$) and PF1&2 levels ($p<0.001$) were still statistically significantly increased in the group of patients with malignancy when the results were age adjusted.

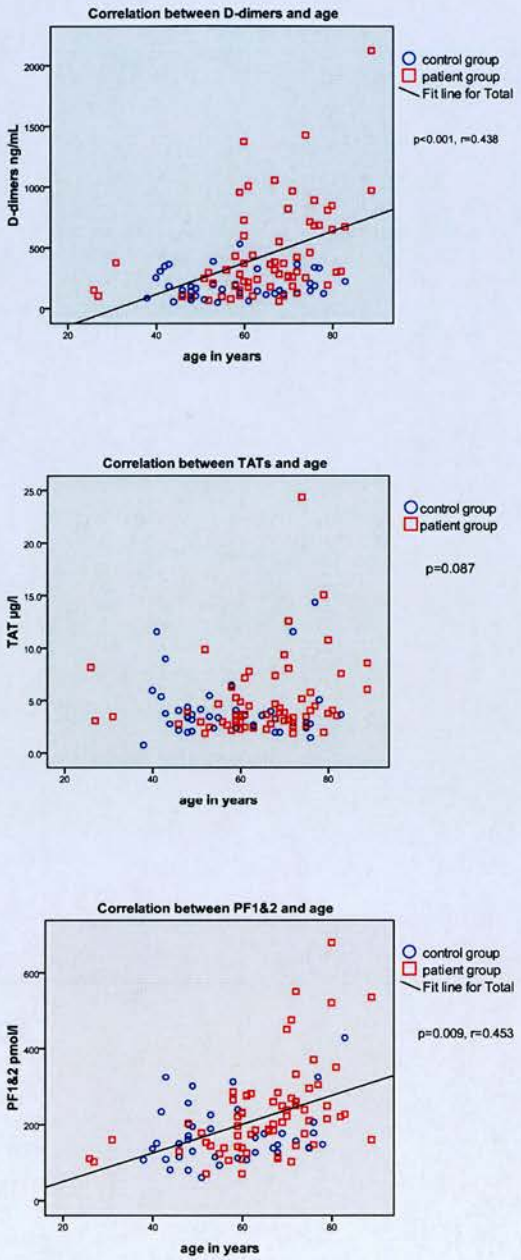


Figure 4.13: Scatterplots showing the relation between the markers of haemostatic activation and age in years (Spearman's correlation coefficient).

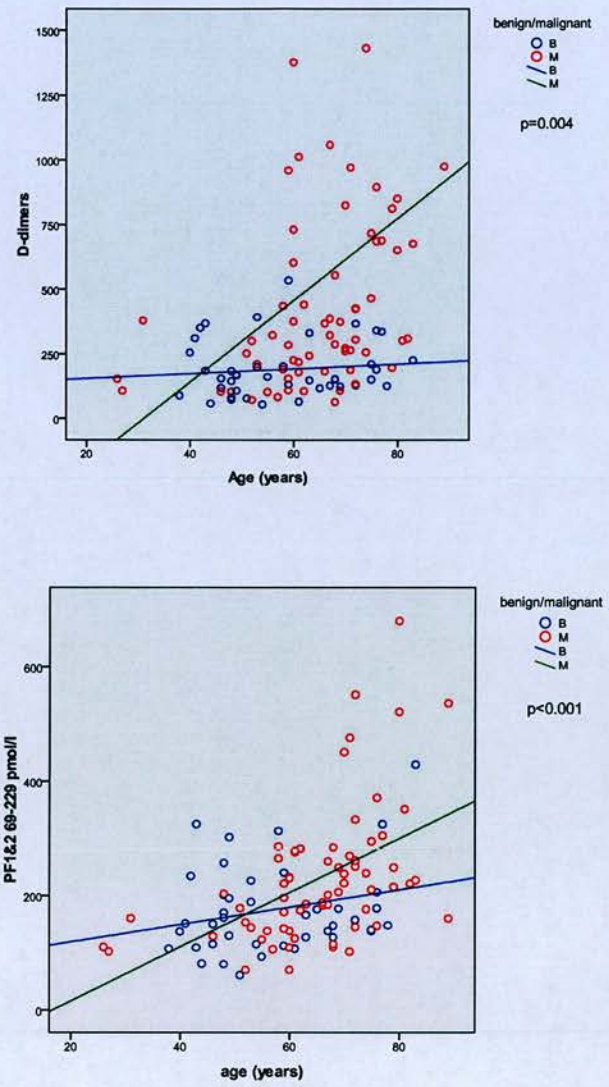


Figure 4.14: Scatterplots showing the age adjusted correlation between the markers of haemostatic activation and malignancy.

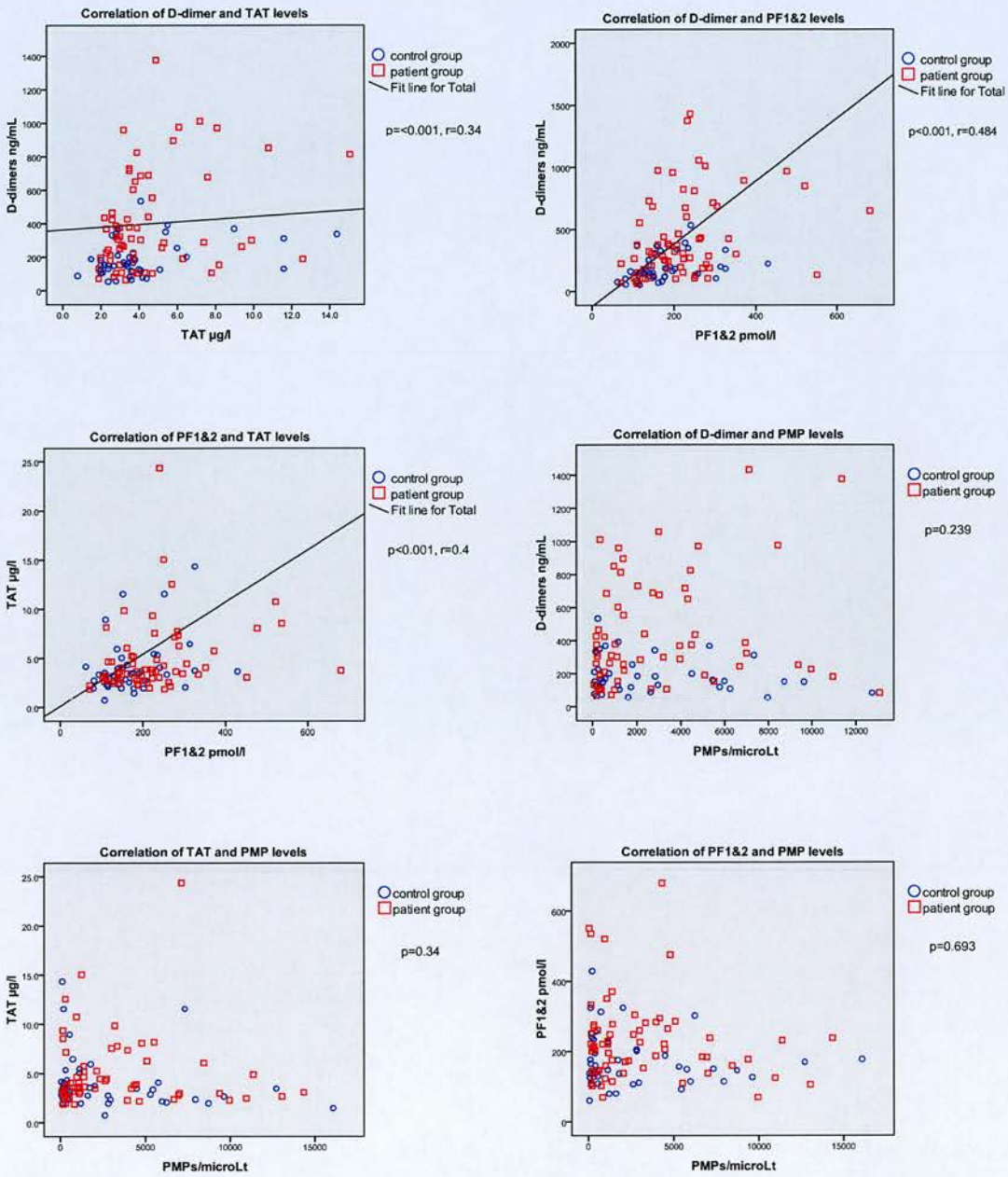


Figure 4.15: Correlation of the levels of D-dimers, PF1&2, TAT and the number of circulating microparticles (MPs) (Spearman's correlation coefficient). There was a weak correlation amongst the level of D-dimers, TAT and PF1&2, but no correlation between the levels of the markers of haemostatic activation and the number of circulating microparticles.

4.4.15 Correlation of markers of haemostatic activation and MPs

There was a weak correlation amongst the levels of D-dimers, TAT and PF1&2. However there was no correlation between the level of D-dimers, TAT and PF1&2 and the number of circulating MPs in individual patients – Figure 4.15, page 115.

4.4.16 Markers of haemostatic activation by diagnosis

There was no statistically significant difference in the measured levels of D-dimers, TAT and PF1&2 between patients with different types of gynaecological malignancy (i.e. cervical, endometrial, ovarian, vulval and vaginal cancer) and between control patients with different reasons for requiring benign gynaecological surgery (i.e. pelvic floor prolapse, benign ovarian cysts, benign fibroids). p value ranging from 0.089 to 0.764, Kruskall Wallis test – please refer to Figure 4.16.

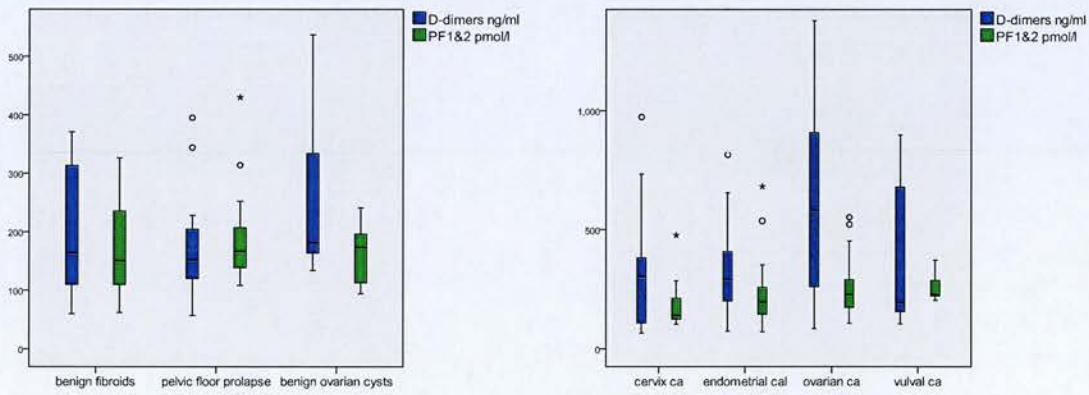


Figure 4.16: Box plots of the level of D-dimers and PF1&2 by specific diagnosis. The results of the single patient with vaginal cancer have not been included. TAT results are also not shown due to the requirement of a very different scale to show these results. There was no significant difference between the patient subgroups (p=0.089-0.764).

4.4.17 Markers of haemostatic activation and thrombosis

Five women from the malignant group and none from the control group were diagnosed with a VTE event during the follow up period (median follow up of 11 months, range of 5-17 months) – Table 4.4, page 89. There was a statistically significant increase in the D-dimer levels in the patients with a VTE (p=0.007, Mann Whitney *U* test), but no statistically significant difference in the level of TAT and PF1&2 between patients with/without a VTE (p=0.183, p=0.078 respectively, Mann Whitney *U* test).

4.4.18 Markers of haemostatic activation and mortality

The level of D-dimers was statistically significantly increased in the 4 patients with gynaecological malignancy that died during the study follow up period (p value 0.003, Mann Whitney *U* test). There was no significant difference in the level of TAT and PF1&2 between patients who died and patients who were alive at the end of the follow up period (p value 0.25 and 0.322 respectively, Mann Whitney *U* test).

4.4.19 Effect of treatment on markers of haemostatic activation

Seven of the women with gynaecological malignancy provided a second set of blood samples on their last day of chemotherapy and/or radiotherapy treatment. The demographics of these 7 patients are summarised in Table 4.9, page 104. There was no statistically significant difference between the level of D-dimers, TAT and PF1&2 measured prior to starting treatment and at the end of treatment (p value ranging from 0.176 to 0.735, Wilcoxon Signed Ranks test) - Figure 4.17, page 118.

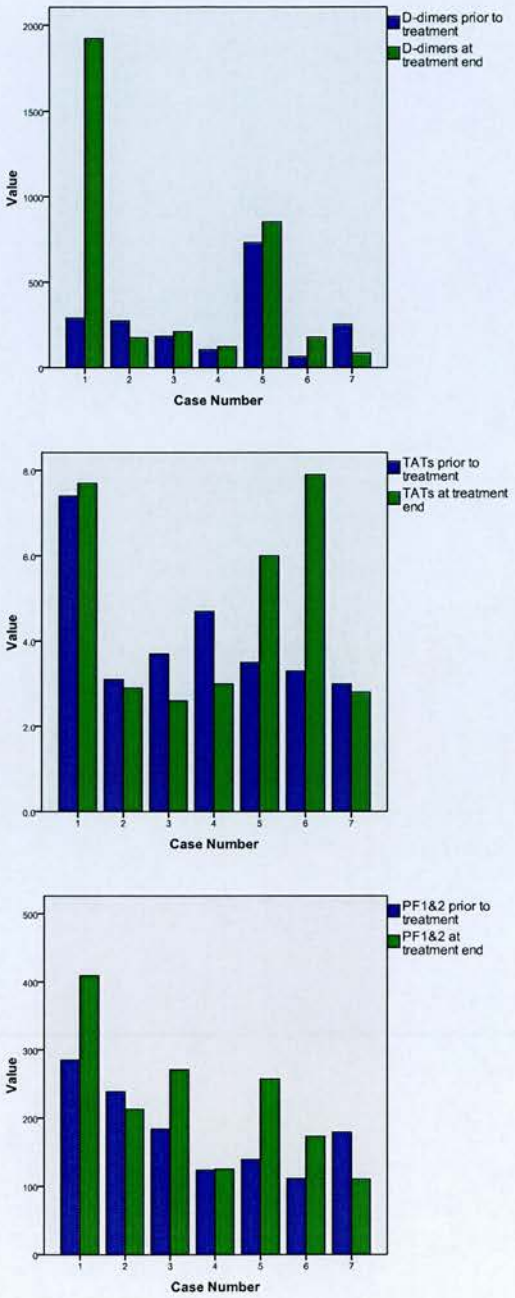


Figure 4.17: Bar charts comparing the level of D-dimers, TAT and PF1&2 prior to starting treatment and at the end of treatment in patients with gynaecological malignancy. There was no significant difference in the paired results of individual patients (p=0.176-0.735)

4.4.20 Markers of haemostatic activation pre- and post-operatively

Seven patients with gynaecological malignancy and 18 women from the control group provided a second blood sample 24 hours after surgery. The level of D-dimers was statistically significantly increased postoperatively compared to the preoperative results ($p<0.001$, Wilcoxon Signed Ranks test). There was no significant difference in the level of TAT and PF1&2 between pre- and post-operative samples (p value 0.277 and 0.94 respectively, Wilcoxon Signed Ranks test) – please refer to Figure 4.18, page 120.

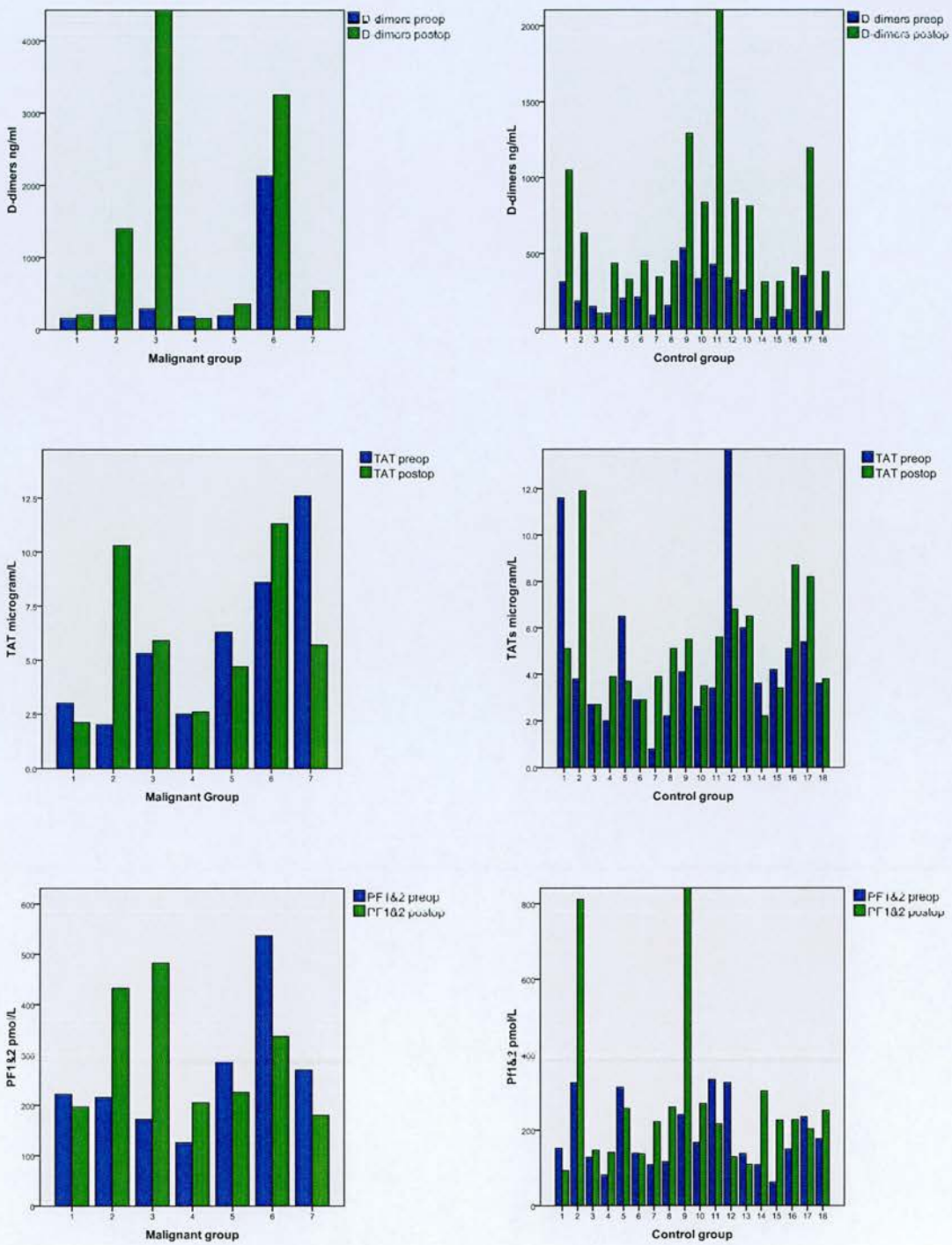


Figure 4.18: Comparison of the level of the markers of haemostatic activation preoperatively and postoperatively. The level of D-dimers was statistically significantly increased postoperatively ($p<0.001$). There was no significant difference in the level of TAT and PF1&2 between the pre- and post-operative results ($p=0.277$ and 0.94).

4.5 DISCUSSION

I recruited a total of 67 women with gynaecological malignancy and a control group of 42 women with no underlying malignancy over a 12 month period (2009), and measured the number of circulating MPs in the two groups of women using light-scattering flow cytometry.

4.5.1 Demographics

The women in the control group tended to be younger than the women in the malignant group, however with a similar age distribution. This difference in median age is likely to reflect the fact that gynaecological malignancy is commoner in older women. On the other hand symptomatic benign gynaecological conditions such as pelvic floor prolapse are more likely to be managed surgically in younger patients, and conservatively in older women who are more likely to have co-morbidities. This age difference is however unlikely to have made a difference to the MP results, there being no correlation between the patients' age and MP numbers measured.

The women in the malignant group tended to have a slightly lower haemoglobin and higher white cell count than the women in the control group, while there was no significant difference in the peripheral blood platelet count between the two groups. Some of the women in the malignant group also had slightly impaired renal function with a reduced EGFR, although in the majority of patients the renal function was normal. These differences in the full blood count and renal function between the two groups are an expected finding in patients with underlying malignancy and may be due to a variety of reasons such as bleeding, the effect of chemo/radiotherapy and renal obstruction by the tumour. I found no correlation between the level of haemoglobin, white cell count and EGFR results and the number of circulating MPs so that these differences between the two groups are unlikely to have influenced the MP results. There was a weak correlation between the patients' peripheral blood platelet count and the number of PMPs measured. However as there was no overall difference in the number of platelets in the peripheral blood between the two patient groups this has not influenced the MP results.

4.5.2 MP results

The number of circulating MPs (all subtypes) in the patients with gynaecological malignancy was very similar to the number of MPs in the control group who had no malignancy. The total number of TF+ve MPs was also very similar between the two patient groups. As far as I can establish this is the first study looking specifically at patients with gynaecological malignancy. My results are different to a number of studies[164, 165] published earlier where investigators have reported increased numbers of circulating MPs in patients with various prothrombotic conditions including malignancy. However more recent studies[23, 81, 159] have, like me, found very similar numbers of circulating MPs in patients with malignancy compared to healthy individuals.

Some investigators[18, 81, 165] have reported increased levels of TF+ve MPs in patients with malignancy, while others found similar numbers of circulating TF+ve MPs but increased TF+ve MP-dependent function[116, 159]. There are now a number of published studies where the results of MP assays using different techniques (i.e. flow cytometry, ELISA and functional assays) do not correlate, underlying the fact that these assays are not interchangeable. For example Tesselaar et al[116] reported similar levels of TF+ve MPs in healthy controls and patients with metastatic cancer, but increased MP dependent TF activity. Similarly Haubold et al[159] found significantly increased TF activity in the cancer patient group, but the TF antigen levels and TF+ve MP levels did not correlate with TF activity. It is possible that I did not find a difference in the number of circulating TF+ve MPs between patients with malignancy and the control group because I measured the TF+ve MPs by flow cytometry. The latter measures the number of events but does not give any information as to the concentration of TF on the individual MPs or whether the TF being detected is active or not. It is hypothesised that functional assays of MPs may better reflect their patho-physiological effect. In the future performing parallel assays looking at both the number of MPs present and the MP-dependent activity may be more informative.

There is a correlation between the number of circulating MP subtypes in individual patients. Patients with increased numbers of PMPs also tended to have increased

levels of EMPs and LMPs, and vice versa. This was true for both the patients with gynaecological malignancy and the control group of women. This may reflect a generalised stimulus leading to MP release from various different types of cells, or may reflect a reduced rate of excretion of MPs in an individual.

PMPs and LMPs together made up the majority of circulating MPs, with EMPs making up a smaller percentage (see Table 4.5, page 91). This is different from the relative proportions of MP subtypes detected by other investigators. There are a number of publications[71, 76, 77, 79, 204] where PMPs make up the majority of circulating MPs. On the other hand Shah[78] found EMPs to be the predominant subtype of circulating MPs. These differences may partly reflect the underlying medical condition in the various patient groups. However since both the malignant group and the control group in this study had very similar proportions of MP subtypes, these differences in the published literature and in my study could also be the result of different handling and processing of samples for MP analysis.

I measured the total number of circulating AV+ve MPs, but was unable to establish their cell of origin, mainly due to the binding buffer needed for AV binding interfering with the monoclonal antibodies binding to MPs. When I compared the total number of AV+ve MPs, with the total number of cell specific MPs i.e. PMPs+EMPs+LMPs, in the vast majority of patients AV+ve MPs made up a much smaller number than the total number of cell-specific MPs (PMPs+EMPs+LMPs). In the majority of patients in this study most of the MPs being measured were AV negative. A number of investigators still use AV positivity as one of the defining criteria when identifying MPs. However this would have resulted in the majority of MPs being missed in this study. There are now a number of published reports[87, 98] indicating that AV negative MPs make up a significant proportion of all circulating MPs. Care must therefore be taken when comparing the results from different investigators to establish the exact criteria used to identify MPs. On the other hand in a small number of the study patients the number of AV+ve MPs was greater than the total number of cell-specific MPs measured (PMPs+EMPs+LMPs) in the individual. Such patients must have a significant number of MPs being released by other cell types such as red cells or tumour cells.

4.5.3 MPs and thrombosis

Out of the 67 women with gynaecological malignancy, 5 (7.5%) were diagnosed with a VTE event within the follow up period (median follow up of 11 months, range 5-17 months). An incidence of 7.5% of VTE is not dissimilar to the quoted incidence rates in the literature of VTE in patients with gynaecological malignancy[8, 9, 23, 25, 44]. Three out of the 5 episodes of VTE occurred in patients with ovarian cancer (12.5% of the patients with ovarian cancer), the latter group of patients often being considered to have the highest risk of thrombotic events in patients with gynaecological malignancy[20]. The other 2 VTE events occurred in patients with vulval cancer (40% of the patients with vulval cancer). None of the patients with cervical cancer, endometrial cancer or vaginal cancer was diagnosed with a thrombotic event.

Four of the VTE events occurred on diagnosis of the underlying malignancy suggesting that the prothrombotic state was a manifestation of the malignancy itself secondary to a physical and/or systemic effect of the tumour. All four patients who presented with a VTE at the same time as their malignancy was diagnosed were only eligible for palliative treatment, either due to generalised frailty of the patient or because of tumour extent. The fifth VTE event occurred on treatment with Carboplatin and Paclitaxel. Treatment with platinums is a well known risk factor for thrombosis[223-225], and Paclitaxel has also been linked with an increased incidence of thrombosis[226, 227].

The absolute number of patients diagnosed with a VTE event was too small to look at VTEs in any formal statistical analysis; however there was no apparent trend between the occurrence of thrombosis and the number of circulating MPs. The power calculations done prior to study recruitment had been based on approximately 25% of women being diagnosed with a VTE at some point during their investigation and treatment for cancer, as per previous publications[7-9] of VTE incidence in patients with gynaecological malignancy. At the interim statistical analysis carried out after 12 months of recruiting patients it was clear that the incidence of VTE events in the study patients was significantly lower at 7.5% (this was later confirmed to be representative of the overall population of patients diagnosed with

gynaecological malignancy in SCAN; see Chapter V). This meant that it would have necessitated a much larger study group than originally planned in order to identify any difference between patients with a VTE and patients with no VTE. It was therefore decided to stop recruiting patients at that point, particularly as there was also no difference in MP numbers between patients with cancer and patients without cancer. It may be that had I continued to recruit patients and/or followed them up for longer (so that potentially more would have been diagnosed with a VTE event) I may have identified a difference in the number of MPs between patients with/out a VTE event.

None of the women in the control group were diagnosed with a thrombotic event within 3 months of surgery. This is likely to reflect the routine use of TED stockings and thromboprophylaxis with low molecular weight heparin perioperatively in all patients undergoing major gynaecological surgery. A number of studies have been published confirming that such thromboprophylaxis is protective against perioperative VTE events[228].

4.5.4 MPs and prognosis

Of all the patients in this study 4 of the women with gynaecological malignancy (6%) and none of the women in the control group died during the follow up period (median 11 months, range 5-17 months). The numbers are too small for formal statistical analysis, however there was no difference in MP numbers in the patients that died. Of the 4 patients who died, two had also been diagnosed with a VTE event, so that 40% of patients with both gynaecological malignancy and a VTE event died during the study period, compared to 3.2% of women with gynaecological malignancy but no thrombosis. The number of patients is too small to draw firm conclusions; however it is suggestive that patients with malignancy who also have a venous thrombotic event have a worse outcome compared to patients with a similar malignancy but no associated thrombosis. Similar findings have been reported by other investigators[6, 12, 19, 20]. It is currently unclear whether the worse prognosis is simply a result of the presence of a VTE (unlikely since the majority of VTE events are not fatal), or whether the occurrence of a VTE in a patient with malignancy is a manifestation of a more aggressive tumour.

4.5.5 MPs and treatment

The treatment in different patients varied according to the specific type of gynaecological malignancy, disease stage and grade and any co-morbidities present. Seven of the patients with gynaecological malignancy provided a second blood sample on their last day of treatment with chemotherapy and/or radiotherapy (week 5 or 6 of treatment, please refer to Pg 78). There was no significant difference in the numbers of circulating MPs in individual patients between blood samples taken prior to starting treatment and blood samples taken at the end of treatment. There was however a trend for the level of PMPs, EMPs and LMPs to be lower at the end of treatment (the results of TF+ve MPs and AV+ve MPs were very variable with no overall trend). At the same time these patients had lower circulating platelet and white cell counts in their peripheral blood at the end of treatment compared to the number of peripheral blood platelets and white cells prior to starting treatment. It may be that the trend for the PMPs, LMPs and EMPs to be lower at the end of treatment may partly reflect the lower number of peripheral blood platelet and white cells, or may be due to the effect that chemo- and/or radio-therapy may have on the rate of MP release. One could also hypothesise that the lower number of MPs detected may be partly due to MPs being recruited to sites of inflammation secondary to the effects of chemoradiotherapy, leaving fewer MPs in the circulation. The numbers are however too small to draw firm conclusions. There was also no significant difference in the level of the markers of haemostatic activation between the blood samples taken prior to treatment and the blood samples at the end of treatment.

4.5.6 Markers of haemostatic activation

I measured the level of D-dimers, TAT and PF1&2 as indirect markers of haemostatic activation. Their levels have been reported to be increased in patients with malignancy and other prothrombotic conditions[20, 170]. In my group of patients there was a statistically significant increase in the level of D-dimers and PF1&2 in patients with gynaecological malignancy even after adjusting for age, and a statistically significant weak correlation between the level of D-dimers, TAT and PF1&2. However there was no correlation between the level of the markers of

haemostatic activation and the number of circulating MPs. This is in keeping with the fact that I found no difference in the number of circulating MPs between the two patient groups.

The results in the literature are variable. Hron et al[81] found a correlation between the number of circulating TF+ve PMPs and the level of D-dimers. However several other investigators have failed to show a correlation between the number of circulating MPs and the level of markers of haemostatic activation[79, 140]. This variability in results may be due to the use of different methods to analyse the number of circulating MPs by different investigators. Given the role MPs are thought to play in coagulation activation and propagation it would be reasonable to expect that the number of circulating MPs and the level of the markers of haemostatic activation would be correlated. The current variability in results with most investigators failing to show a correlation is likely to partly reflect the lack of standardisation of MP assays. It may also be that measuring MP-dependent activity rather than MP numbers would better reflect the role of MPs in coagulation activation. The relationship between the level of circulating MPs (and/or their function) and the markers of haemostatic activation needs to be re-assessed once the assays for MP analysis are standardised.

There was a statistically significant increase in the level of D-dimers in the patients who had had a thrombotic event, reflecting haemostatic activation with fibrin generation in these patients. There was also a statistically significant increase in the level of D-dimers in the 4 patients that died during the follow up period. Two of these 4 patients had also been diagnosed with a thrombotic event. Increased levels of haemostatic activation secondary to the presence of advanced malignancy is likely to have contributed to the increased levels of D-dimers in these 4 patients.

In a number of patients I measured the number of MPs and the markers of haemostatic activation both pre- and post-operatively. The number of circulating MPs postoperatively was very variable and difficult to interpret with no particular trend. On the other hand there was a statistically significant increase in the level of D-dimers in the postoperative samples. The latter can be explained due to the trauma

of surgery and the necessary haemostatic activation to control bleeding postoperatively.

4.5.7 Limitations of the study

This is a single centre relatively small study including patients with all types of gynaecological malignancy. This has lead to a heterogeneous population of cancer patients, different subtypes of gynaecological malignancy having different prognoses and being associated with different degrees of thrombotic risk. The absolute number of study patients diagnosed with a thrombotic event is too small to allow generalised conclusions regarding the prothrombotic nature or otherwise of measured MPs.

I have analysed MPs using light-scattering flow cytometry. This is currently the commonest technique to characterise MPs. However it has a number of limitations, mainly due to the small size of MPs so that the MPs appear close to the electronic noise of current flow cytometers. This makes their accurate identification difficult. Further, flow cytometry does not given any information as to the functional activity of the measured MPs. There is considerable research currently taking place with the aim of making the identification of MPs more specific and accurate.

The variability in the results from different investigators is likely to be partly due to the lack of a standardised method by which to identify and measure MPs. There are still many variables and problems, not least the small size of MPs which challenges the currently available technology, and the lack of a universal definition of what constitutes a MP. Comparing results between different investigators is therefore difficult. The different protocols used by different laboratories to analyse MPs are likely to significantly affect the absolute number of MPs measured. In this study all the patient samples were analysed by a single operator, using a tightly controlled protocol, so that although the results from this study may not be comparable with the results from other laboratories using a different technique, they are comparable for this cohort of patients.

One of the main reasons for result variability between different laboratories is the use of different centrifugation techniques with the risk of either removing MPs or

"generating" MPs by fragmentation during sample processing. In view of the real concern of "generating" MPs during ultra-centrifugation I decided to instead use serial centrifugation to prepare PFP. The centrifugation protocol I used in my study produced PFP with a residual platelet count of $5x10^9$/L or less in the majority of samples. I still however had a few patient samples with detectable residual platelets in the PFP, albeit at low numbers. There was a statistically significant strong correlation between the residual platelet count in the PFP and the number of PMPs measured by the flow cytometer. This is likely to be due to the residual platelets interfering with PMP identification such that small platelets (<1 μm) were being measured in the MP gate by the flow cytometer, underlying the current problems with the available technology. The cut-off of 1 μm for the upper size limit of MPs is arbitrary so that some MPs will be larger than 1 μm and by using my method of gating on size some of the larger MPs will be missed. However it is difficult to resolve this issue at present since both platelets and MPs are on a continuum of size and some platelets will be <1 μm and some MPs will be larger than 1 μm, making the accurate and specific measurement of MP numbers difficult.

4.6 CONCLUSION

I found no difference in the number of circulating PMPs, EMPs and LMPs between the group of patients with gynaecological malignancy and the control group. There was also no difference in the number of circulating TF+ve MPs. The incidence of VTE events in the study cohort was 7.5%, similar to the overall incidence of VTEs in patients with gynaecological malignancy in SCAN (please refer to Chapter V). The absolute number of patients with thrombosis was too small to be able to make generalised conclusions, however there was no statistically significant difference in the number of MPs in the patients with gynaecological malignancy who also had a VTE. This means that MP numbers cannot at present be used to help identify patients with malignancy at a higher risk of venous thrombotic events.

CHAPTER V

COHORT OF PATIENTS DIAGNOSED WITH GYNAECOLOGICAL MALIGNANCY IN THE SOUTH EAST OF SCOTLAND CANCER NETWORK IN 2009

A DESCRIPTIVE STUDY

5.1 INTRODUCTION

I wanted to establish whether the cohort of patients recruited to the study on MPs was representative of the overall population of patients being diagnosed with gynaecological malignancies in the South East of Scotland Cancer Network (SCAN), in order to determine whether the results of the MP study could be extrapolated to the rest of the patients with gynaecological malignancy in SCAN.

I have therefore carried out an audit of all the patients presenting to SCAN with a confirmed gynaecological malignancy over one calendar year (2009 - the same time period during which I recruited patients to the MP study) in order to establish the overall incidence of the different gynaecological malignancies and the incidence of thromboses in this patient group. Here I describe the demographics and clinical characteristics of these patients, and correlate my audit findings with the demographics of the MP study cohort.

5.2 Methods

5.2.1 Patient Identification

All patients who are likely to have a diagnosis of gynaecological malignancy are discussed in a weekly gynae-oncology multidisciplinary meeting (MDM) in SCAN. I was therefore able to identify all patients with a confirmed diagnosis of gynaecological malignancy from the weekly MDM discussion lists for the relevant period of time. The audit was registered with the Clinical Governance Support Team. Prior to commencing data collection, permission was sought and obtained from the chairperson of the SCAN gynae-oncology multidisciplinary team and all the gynaecology and oncology consultants who treat patients with gynaecological malignancy.

5.2.2 Data collection

The list of patients discussed in the weekly gynae-oncology MDM meetings during 2009 was obtained from the MDM co-ordinator. Patient details, including tumour site, histological diagnosis, disease stage, treatment, past medical history, evidence of VTE (DVT and/or pulmonary embolism) or arterial events, smoking history and overall outcome were collected by going through the patients' medical records on the Lothian NHS IT system.

5.2.3 Statistics

The audit data was analysed and the descriptive statistics are reported in this chapter. The findings have been compared between patient subgroups using the Mann Whitney *U* test, with two-tailed significance testing. Statistical significance was defined as a p value of < 0.05. Analyses were performed with the statistical package SPSS version 17.0 (SPSS Inc, Chicago, IL, USA).

5.3 Audit Findings

5.3.1 Patient details

During 2009 a total of six hundred and eighty one (681) patients were discussed at the gynae-oncology MDM meetings. Of these patients five hundred and eighty four (584) were confirmed to have a current diagnosis of gynaecological malignancy or gynaecological pre-malignant condition (CIN, VIN, VAIN or borderline ovarian tumours). Another 17 patients had a previous history of gynaecological malignancy but no recurrence was confirmed during 2009. A further 80 patients did not have a diagnosis of gynaecological malignancy confirmed, often having instead a benign gynaecological condition or a malignancy arising elsewhere.

The population living in SCAN is made up of approximately 1.45 million people, 0.7 million of whom are women (General Register Office for Scotland mid-2009 population estimate)[3]. The patients treated by the SCAN team are referred from a large geographical area which includes the Lothian area, as well as Dumfries & Galloway, The Borders and Fife. All these patients are discussed in the weekly gynae-oncology SCAN MDM so that treatment is uniform across the whole region. The details of all patients managed by the SCAN team are recorded electronically on the Lothian NHS IT system. The data for the audit (including diagnosis, treatment planned and complications) was gathered by going through the individual patients' medical records on this computer system. This computer system is however not linked up to hospitals outside Lothian i.e. Dumfries & Galloways, The Borders and Fife. Patients with gynaecological malignancy living in the latter geographical areas of SCAN are still managed by the same multidisciplinary team for their malignancy, however in the case of an acute emergency such as a DVT or pulmonary embolism, they would normally present to their local hospital.

Of the 584 patients with a confirmed diagnosis of gynaecological malignancy referred to SCAN, 375 were living in Lothian i.e. these patients would use one of the hospitals within Lothian as their local hospital. For this group of patients (group A) I had access to all their medical records i.e. detailing both the management of their gynaecological malignancy and any other reason for attending hospital. The remaining 209 patients lived outside Lothian (group B). For these patients I had

access to their medical records detailing the management of their malignancy, however I did not have access to their records detailing acute admissions to their local hospitals for the management of complications such as DVT/pulmonary embolism. In the majority of cases I was still able to ascertain the occurrence of such events by reviewing all correspondence sent to the oncology team. However, there is the possibility that some such events will have been missed purely because of a lack of such correspondence, for example in patients who had already finished treatment for their malignancy, or patients who were being palliated locally and no longer requiring active treatment. In view of this I have analysed the data I gathered for the whole patient group (n=584), and also analysed the two subgroups of patients separately (patients living within Lothian (n=375 – group A) and patients living outside Lothian (n=209 – group B)) to establish whether there were any significant differences in the documented occurrence of complications (such as VTEs) between the two groups of patients and thus determine how robust my data collection has been.

5.3.2 Patient demographics

The patient demographics of the women diagnosed with gynaecological malignancy or a gynaecological pre-malignant condition (CIN, VIN, VAIN, borderline ovarian tumours) in SCAN in 2009 are detailed in Table 5.1, page 135. Of the 584 patients diagnosed with gynaecological malignancy, 504 (86%) were new diagnoses, while the remaining 80 (14%) had a history of gynaecological malignancy diagnosed in the past and had re-presented again in 2009 with relapsed disease. The relative proportions did not change when I considered only the 375 patients living within Lothian (324 (86%) new diagnoses and 51 (14%) with a history of gynaecological malignancy re-presenting in 2009), or the 209 patients living outside Lothian (180 (86%) new diagnoses and 29 (14%) with a history of gynaecological malignancy re-presenting in 2009). The pathological subtypes of the different gynaecological malignancies are presented in Table 5.2 a-e, page 136-138.

Gynaecological malignancy site	Total no. of pts n=584 (% of 584)	Pt group living within Lothian (A) n=375 (% of 375)	Pt group living outside Lothian (B) n=209 (% of 209)	group A v.s. group B p value
Endometrial	215 (37%)	135 (36%)	80 (38.3%)	n/s
Ovarian Borderline ovarian	191 (32.7%) 9 (1.5%)	112 (29.9%) 8 (2.1%)	79 (37.8%) 1 (0.5%)	n/s
Cervical CIN	89 (15.2%) 16 (2.7%)	62 (16.5%) 13 (3.5%)	27 (13%) 3 (1.4%)	n/s
Vulval VIN	48 (8.2%) 4 (0.7%)	35 (9.3%) 2 (0.5%)	13 (6.2%) 2 (0.96%)	n/s
Vaginal VAIN	5 (0.9%) 2 (0.3%)	4 (1.1%) 1 (0.3%)	1 (0.5%) 1 (0.5%)	n/s
Unknown primary	3 (0.5%)	1 (0.26%)	2 (0.96%)	n/s
Peritoneal mesothelioma	1 (0.17%)	1 (0.26%)	0	n/s
Bartholin's gland	1 (0.17%)	1 (0.26%)	0	n/s

Table 5.1: Details of the number of patients diagnosed with the different subtypes of gynaecological malignancy. Comparison is made between the whole patient group diagnosed with gynaecological malignancy treated in SCAN, and the two patient subgroups according to geographical areas i.e. patient group A, living within Lothian and patient group B, living outside Lothian, but still in SCAN. The patient subgroups have a very similar incidence of the different subtypes of gynaecological malignancies so that the group living within Lothian is representative of the whole patient cohort overall. p value for 2-tailed significance testing was calculated using the Mann Whitney *U* test for 2 independent samples. Cervical intraepithelial neoplasia (CIN), vulval intraepithelial neoplasia (VIN), vaginal intraepithelial neoplasia (VAIN), number (no.), not statistically significant (n/s), patient (pt).

Cervical malignancy: histological diagnoses	
Histological subtype	**Number of patients n=105 (%)**
Squamous carcinoma	66 (62.9%)
Adenocarcinoma	14 (13.3%)
Neuroendocrine carcinoma	2 (1.9%)
Endometrioid carcinoma	2 (1.9%)
Adenosquamous carcinoma	1 (0.95%)
Clear cell carcinoma	1 (0.95%)
Serous carcinoma	1 (0.95%)
CIN	16 (15.2%)
Information missing	2 (1.9%)

Table 5.2a: Cervical malignancy patients – histological subtypes. Cervical intra-epithelial neoplasia (CIN)

Endometrial malignancy: histological diagnoses	
Histological subtype	**Number of patients n=215 (%)**
Endometrioid carcinoma	156 (72.6%)
Serous papillary carcinoma	26 (12.1%)
Carcinosarcoma	10 (4.7%)
Clear cell carcinoma	5 (2.3%)
Leiomyosarcoma	5 (2.3%)
Stromal sarcoma	3 (1.4%)
Squamous carcinoma	1 (0.5%)
Information missing	9 (4.2%)

Table 5.2b: Endometrial malignancy patients – histological subtypes.

Ovarian malignancy: histological diagnoses	
Histological subtype	**Number of patients n=200 (%)**
Serous carcinoma	83 (41.5%)
Mucinous carcinoma	22 (11%)
Endometrioid carcinoma	19 (9.5%)
Primary peritoneal adenocarcinoma	17 (8.5%)
Clear cell carcinoma	10 (5%)
Granulosa cell carcinoma	7 (3.5%)
Fallopian tube serous papillary carcinoma	3 (1.5%)
Malignant teratoma	3 (1.5%)
Carcinosarcoma	2 (1%)
Cystadenocarcinoma	1 (0.5%)
Pseudomyxoma peritonei	1 (0.5%)
Borderline ovarian tumours	9 (4.5%)
Information missing	23 (11.5%)

Table 5.2c: Ovarian malignancy patients – histological subtypes.

Vulval malignancy: histological diagnoses	
Histological subtype	**Number of patients n=52 (%)**
Squamous carcinoma	43 (82.7%)
Melanoma	3 (5.8%)
Verrucous carcinoma	1 (1.9%)
Basal cell carcinoma	1 (1.9%)
VIN	4 (7.7%)

Table 5.2d: Vulval malignancy patients – histological subtypes. Vulval intra-epithelial neoplasia (VIN)

Miscellaneous: histological diagnoses	
Cancer site	**Histological diagnosis (n)**
Vaginal malignancy	Squamous carcinoma (n=5)
	VAIN (n=2)
Unknown primary	Squamous carcinoma (n=1)
	Neuroendocrine carcinoma (n=1)
	Adenocarcinoma (n=1)
Bartholin's gland ca	Adenoid cystic carcinoma (n=1)
Peritoneal mesothelioma	Mesothelioma (n=1)

Table 5.2e: Miscellaneous gynaecological malignancies - histological subtypes. Vaginal intra-epithelial neoplasia (VAIN), cancer (ca)

5.3.3 Management of gynaecological malignancies

The treatment offered to the different patients varied according to the specific diagnosis and histological subtype, disease stage, any previous treatment and pre-existing patient co-morbidities. Many of the treatment modalities used in patients with gynaecological malignancy contribute to a prothrombotic state e.g. surgery and chemotherapy, especially Cisplatin[223-225]. I have therefore summarised the different treatment modalities used according to cancer type (see Table 5.3a-f, page 139-143) to assess whether there was a particular treatment associated with a significantly increased incidence of venous or arterial events (see later).

Cervical malignancy n=105		
Treatment	**Details of chemotherapy**	**Number of patients**
Chemotherapy	Cisplatin & Etoposide	1
	Cisplatin & Topotecan	2
	Information missing	1
Chemotherapy & radiotherapy	Cisplatin	32
	Cisplatin & Topotecan	2
Surgery only	n/a	26
Surgery & chemotherapy	Cisplatin & Topotecan	1
Surgery, chemotherapy & radiotherapy	Cisplatin	6
	Carboplatin & Paclitaxel	1
Radiotherapy only	n/a	12
LETZ	n/a	18
Patient declined treatment	n/a	3

Table 5.3a: Summary of the treatments used in patients with cervical malignancy.

Endometrial malignancy n=215		
Treatment	**Details of chemotherapy**	**Number of patients**
Chemotherapy only	Liposomal Doxorubicin	1
	Carboplatin	3
	Carboplatin & Paclitaxel	3
Chemotherapy & radiotherapy	Carboplatin	1
Surgery only	n/a	90
Surgery & chemotherapy	Carboplatin & Paclitaxel	10
	Carboplatin	2
	Liposomal Doxorubicin	1
	Carboplatin, Paclitaxel, Liposomal Doxorubicin	1
Surgery, chemotherapy & radiotherapy	Carboplatin & Paclitaxel	17
	Carboplatin	2
Surgery & radiotherapy	n/a	58
Hormone treatment	n/a	11
Radiotherapy only	n/a	3
Patient declined treatment	n/a	4
Unfit for treatment	n/a	8

Table 5.3b: Summary of the treatments used in patients with endometrial malignancy.

Ovarian malignancy n=200		
Treatment	**Details of chemotherapy**	**Number of patients**
Surgery only	n/a	46
Surgery & chemotherapy	Carboplatin & Paclitaxel	48
	Carboplatin	14
	Cisplatin & Etoposide	2
	Carboplatin & Cisplatin	1
	Liposomal Doxorubicin	1
	Carboplatin, Paclitaxel & Bevacizumab	1
Chemotherapy only	Carboplatin	28
	Carboplatin & Paclitaxel	18
	Cisplatin & Etoposide	1
	Topotecan	1
Radiotherapy only	n/a	2
Hormone treatment	n/a	5
Watch & wait	n/a	5
Patient declined treatment	n/a	8
Unfit for treatment	n/a	18
Information missing	n/a	1

Table 5.3c: Summary of the treatments used in patients with ovarian malignancy.

Vulval malignancy n=52		
Treatment	**Details of chemotherapy**	**Number of patients**
Chemotherapy & radiotherapy	Cisplatin	2
Radiotherapy only	n/a	8
Surgery only	n/a	33
Surgery & radiotherapy	n/a	4
Patient declined treatment	n/a	1
Unfit for treatment	n/a	4

Table 5.3d: Summary of the treatments used in patients with vulval malignancy.

Vaginal malignancy n=7		
Treatment	**Details of chemotherapy**	**Number of patients**
Surgery only	n/a	2
Chemotherapy only	Cisplatin	1
Chemotherapy & radiotherapy	Cisplatin	2
Radiotherapy only	n/a	2

Table 5.3e: Summary of the treatments used in patients with vaginal malignancy.

Miscellaneous		
Diagnosis	**Treatment**	**Number of patients**
Unknown primary	Surgery, Carboplatin & Paclitaxel	1
	Surgery only	1
	Radiotherapy only	1
Bartholin's gland tumour	Surgery, Cisplatin & radiotherapy	1
Peritoneal mesothelioma	Surgery only	1

Table 5.3f: Summary of the treatments used in patients with various gynaecological malignancies.

5.3.4 Incidence of thrombosis

I gathered information on the number of patients who were diagnosed with a thrombotic event in the year prior to or concurrent with the diagnosis of malignancy, and also subsequent to this, either while still on treatment or in the follow up period. The data presented is correct as of June 2010 (median follow up of 11 months, range 5-17 months).

In view of the fact that patients living outside Lothian would not normally present to one of the hospitals within Lothian for acute events (i.e. DVT, pulmonary embolism, MI, CVA) I looked at the patient cohort as a whole and also at the two subsets of patients according to geographical location i.e. patients living within Lothian (group A) and patients living outside Lothian (group B), and compared my findings between the two subgroups. In the case of patient group A, i.e. patients living within Lothian, I had easy access to all their hospital records, and I was therefore confident that I had a complete picture of any complications from the malignancy or its treatment. On the other hand in the case of patient group B, i.e. patients living outside Lothian, but still within SCAN, I did not have access to their local hospital medical records. There is therefore the possibility that I may have missing data on the occurrence of thrombotic events. By comparing the two subgroups of patients I was able to establish how robust my data collection for the whole cohort of patients has been. The data is summarised in Table 5.4, page 145-146.

When calculating the incidence of thrombotic events I did not include any patients with a pre-malignant condition, i.e. CIN, VIN, VAIN and borderline ovarian tumours, but focused instead on patients with invasive malignancies. Pre-malignant conditions are normally completely removed surgically, and are not generally considered to be associated with an increased risk of thrombosis. In fact in this cohort none of the patients with a pre-malignant condition were diagnosed with a thrombotic event.

Diagnosis	VTE events on Dx of ca	VTE events post ca Dx	Total VTE events	MI post Dx of ca	CVA post Dx of ca	Total all thrombotic events	p value Total events A vs B
Ovarian ca All pts (n=191)	**19 (9.9%)**	**9 (4.7%)**	**28 (14.6%)**	**3 (1.6%)**	**7 (3.7%)**	**38 (19.9%)**	**n/a**
Ovarian ca Group A (n=112)	9 (8%)	4 (3.6%)	13 (11.6%)	1 (0.9%)	4 (3.6%)	18 (16.1%)	n/s
Ovarian ca Group B (n=79)	*10 (12.7%)*	*5 (6.3%)*	*15 (19%)*	*2 (2.5%)*	*3 (3.8%)*	*20 (25.3%)*	
Endometrial ca All pts (n=215)	**5 (2.3%)**	**3 (1.4%)**	**8 (3.7%)**	**0**	**1 (0.47%)**	**9 (4.2%)**	**n/a**
Endometrial ca Group A (n=135)	3 (2.22%)	1 (0.74%)	4 (2.96%)	0	1 (0.74%)	5 (3.7%)	n/s
Endometrial ca Group B (n=80)	*2 (2.5%)*	*2 (2.5%)*	*4 (5%)*	*0*	*0*	*4 (5%)*	
Cervical ca All pts (n=89)	**1 (1.1%)**	**2 (2.2%)**	**3 (3.3%)**	**1 (1.1%)**	**0**	**4 (4.5%)**	**n/a**
Cervical ca Group A (n=62)	1 (1.6%)	1 (1.6%)	2 (3.2%)	1 (1.6%)	0	3 (4.8%)	n/s
Cervical ca Group B (n=27)	*0*	*1 (3.7%)*	*1 (3.7%)*	*0*	*0*	*1 (3.7%)*	
Vulval ca All pts (n=48)	**2 (4.2%)**	**1 (2.1%)**	**3 (6.3%)**	**0**	**1 (2.1%)**	**4 (8.3%)**	**n/a**
Vulval ca Group A (n=35)	1 (2.9%)	1 (2.9%)	2 (5.7%)	0	0	2 (5.7%)	n/s
Vulval ca Group B (n=13)	*1 (7.7%)*	*0*	*1 (7.7%)*	*0*	*1 (7.7%)*	*2 (15.4%)*	
Vaginal ca All pts (n=5)	**1 (20%)**	**1 (20%)**	**2 (40%)**	**0**	**0**	**2 (40%)**	**n/a**
Vaginal ca Group A (n=4)	1 (25%)	1 (25%)	2 (50%)	0	0	2 (50%)	n/s
Vaginal ca Group B (n=1)	*0*	*0*	*0*	*0*	*0*	*0*	

Diagnosis	VTE events on Dx of ca	VTE events post ca Dx	Total VTE events	MI post Dx of ca	CVA post Dx of ca	Total all thrombotic events	p value Total events A vs B
Bartholin's gland (n=1)	**0**	**0**	**0**	**0**	**0**	**0**	**n/a**
Mesothelioma (n=1)	**0**	**0**	**0**	**0**	**0**	**0**	**n/a**
Unknown primary (n=3)	**0**	**0**	**0**	**0**	**0**	**0**	**n/a**
Total events All pts (n=553)	**28 (5.1%)**	**16 (2.9%)**	**44 (8%)**	**4 (0.72%)**	**9 (1.6%)**	**57 (10.3%)**	**n/a**
Total events Group A (n=351)	15 (4.3%)	8 (2.3%)	23 (6.6%)	2 (0.6%)	5 (1.4%)	30 (8.5%)	n/s
Total events Group B (n=202)	*13 (6.4%)*	*8 (4%)*	*21 (10.4%)*	*2 (0.99%)*	*4 (1.98%)*	*27 (13.4%)*	

Table 5.4: Summary of the incidence of all thrombotic events, including both venous thromboembolic (VTE) events (pulmonary embolism and deep vein thrombosis) and arterial events (myocardial infarctions (MI) and cerebrovascular accidents (CVA)). Results are presented for the whole patient cohort diagnosed with gynaecological malignancy in SCAN, and also for the two patient subgroups according to geographical locations – group A: patients living within Lothian and group B: patients living outside Lothian. There was no statistically significant difference in the incidence of either venous or arterial events between the two subgroups, implying that my data collection for the whole patient group (which was made up of the two patient subgroups) was robust. The results for the whole patient cohort are presented in bold, the results in normal font refer to the group of patients living within Lothian (group A) and the results in italics refer to the group of patients living outside Lothian (group B). Patients with a diagnosis of a pre-malignant condition (CIN, VIN, VAIN and borderline ovarian tumours) have been excluded from this analysis. The data is correct as of June 2010; median follow up of 11 months, range 5-17 months. p value for 2-tailed significance testing was calculated using the Mann Whitney *U* test for 2 independent samples. Diagnosis (Dx), cancer (ca), not applicable (n/a), not statistically significant (n/s)

5.3.5 Correlation of thrombotic events with treatment

I have analysed the audit data to establish whether the incidence of thrombotic events correlates with a specific treatment modality. The results are summarised in Table 5.5, page 148. The highest incidence of thrombotic events (DVT, pulmonary embolism, CVA, MI) was in the group of patients with extensive metastatic disease who were too unfit to be treated (30% (9 of 30) of this group of patients were diagnosed with a thrombotic event). Patients on chemotherapy also had a significant number of thrombotic events (6.5%, 15 of 230), with the highest incidence seen in patients treated with Carboplatin and Paclitaxel in combination (9.8%, 10 of 102) or Carboplatin alone (10%, 5 of 50). Of all the patients undergoing surgery, only 1.1% (4 of 372) were diagnosed with a thrombotic event in the perioperative period (up to 3 months postoperatively), and only one (0.6% (1 of 158)) of all patients being treated with radiotherapy was diagnosed with a thrombotic event during such treatment.

Treatment given (n=no. of pts receiving Rx modality)		Cancer diagnosis	Thrombotic event	No. of pts (total n=29)	Total number of thrombotic events per treatment modality (% of patients receiving Rx modality)	
None - extensive disease, pt unfit for treatment (n=30)		Cervical	VTE	2	All events: 9 (30%) VTE: 5 (16.7%) Arterial events: 4 (13.3%)	
		Cervical	MI	1		
		Ovarian	VTE	1		
		Ovarian	CVA	3		
		Vaginal	VTE	1		
		Endometrial	VTE	1		
Postoperatively (up to 3 months) (n=372)		Endometrial	VTE	1	All events: 4 (1.1%) VTE: 1 (0.3%) Arterial events: 3 (0.8%)	
		Ovarian	MI	2		
		Vulval	CVA	1		
All types of chemotherapy (n=230)	Carboplatin & Paclitaxel (n=102)	Endometrial	VTE	1	10 (9.8%)	For all types of chemotherapy All events: 15 (6.5%) VTE: 9 (3.9%) Arterial events: 6 (2.6%)
		Endometrial	CVA	1		
		Ovarian	VTE	3		
		Ovarian	CVA	4		
		Ovarian	MI	1		
	Carboplatin (n=50)	Ovarian	VTE	5	5 (10%)	
Radiotherapy (n=158)		Vulval	VTE	1	1 (0.6%)	

Table 5.5: Summary of thrombotic events according to treatment given. This table does not include the thrombotic events occurring prior to, or concurrent with, the diagnosis of malignancy. The patient who was diagnosed with a venous thromboembolic (VTE) event while being treated with radiotherapy had very extensive disease and was being treated with palliative radiotherapy to control her symptoms. Her extensive disease is therefore a more likely risk factor for her thrombotic event. Patient (pt), number (no.), treatment (Rx), myocardial infarction (MI), cerebrovascular accident (CVA)

5.3.6 Prognosis in patients with thrombotic events

I analysed the number of patients who died between their initial presentation in 2009 and June 2010 (time of writing). The median length of follow up was 11 months (range 5-17 months). Once again in view of the possibility of having missing information from patients who did not live within Lothian I analysed the data both for the whole group of patients treated in SCAN and also for the two subgroups of patients according to geographical locations (group A living within Lothian, group B living outside Lothian, but still in SCAN), and compared the latter two subgroups to assess how robust my data collection has been.

Of all the patients with a gynaecological malignancy (excluding pre-malignant conditions) treated in SCAN (553 patients), 78 (14.1%) died during the follow up period. Twenty five (32%) of these patients had also had a thrombotic event, 23 (29.5%) a VTE and 2 (2.5%) an arterial event.

Out of the whole group of patients with gynaecological malignancy (excluding pre-malignant conditions) 57 had had a thrombotic event (44 a VTE and 13 an arterial event). Of all the patients who had had a venous thrombotic event 52% died in the follow up period, compared to 15% of patients with an arterial event and 11% of patients with no thrombosis. This suggests that patients with gynaecological malignancy who are also found to have a venous thrombosis may have a worse outcome when compared with patients with gynaecological malignancy but no thrombosis. However on multivariate analysis this finding was not found to be statistically significant (beta=-0.57, p=0.531).

On subgroup analysis, of the 351 patients living within Lothian (group A; excluding pre-malignant conditions), 46 (13.1%) patients died during the follow up period; 15 (32.6%) of these patients had also had a thrombotic event, 14 (30.4%) a VTE and 1 (2.2%) an arterial event. Of all the patients in group A (excluding pre-malignant conditions) 30 had been diagnosed with a thrombotic event, 23 a VTE and 7 an arterial event; of the patients with a VTE, 61% died during the follow up period, compared to 14% of patients with an arterial event and 10% of patients with no thrombosis (Table 5.6, page 151).

Of the 202 patients living outside Lothian (group B; excluding pre-malignant conditions), 32 (15.8%) died during the follow up period; 10 (31.2%) of these patients had also had a thrombotic event, 9 (28.1%) a VTE and 1 (3.1%) an arterial event. Of all the patients in group B (excluding pre-malignant conditions), 27 had been diagnosed with a thrombotic event, 21 a VTE and 6 an arterial event; of the patients with a VTE, 43% died during the follow up period, compared to 17% of patients with an arterial event and 13% of patients with no thrombosis (Table 5.6, page 151).

Subgroup analysis confirms that the two patient subgroups are very similar with no significant difference ($p=0.317$). This means that my data collection is robust, allowing me to conclude that the statistics for the overall patient cohort are truly representative.

Patient group	Thrombotic history	Outcome
Whole patient group n=553	**+ VTE n=44**	**deceased n=23 (52%)**
		alive n=21 (48%)
	+ arterial events n=13	**deceased n=2 (15%)**
		alive n=11 (85%)
	no thrombosis n=496	**deceased n=53 (11%)**
		alive n=443 (89%)
Patient group A n=351	+ VTE n=23	deceased n=14 (61%)
		alive n=9 (39%)
	+ arterial events n=7	deceased n=1 (14%)
		alive n=6 (86%)
	no thrombosis n=321	deceased n=31 (10%)
		alive n=290 (90%)
Patient group B n=202	*+ VTE n=21*	*deceased n=9 (43%)*
		alive n=12 (57%)
	+ arterial events n=6	*deceased n=1 (17%)*
		alive n=5 (83%)
	no thrombosis n=175	*deceased n=22 (13%)*
		alive n=153 (87%)

Table 5.6: Summary of the number of patients who died in the follow up period (median 11 months, range 5-17 months), and correlation with outcome. The data for the whole patient group is shown in bold, in normal font for the subgroup of patients living within Lothian (group A) and in italics for the subgroup of patients living outside Lothian, but still in SCAN (group B). Venous thromboembolism (VTE) refers to deep vein thrombosis and pulmonary embolism, while arterial events refer to cerebrovascular accidents or myocardial infarction. Patients with pre-malignant conditions are excluded from this analysis.

5.3.7 Smoking history

Information about the patients' smoking history was available in just under half of all the patients (n=272). Of these, 81 were smokers (currently smoking or having stopped smoking less than a year prior to cancer diagnosis), 141 were life long non-smokers and 51 were ex-smokers (defined as having stopped smoking at least one year prior to presenting with gynaecological malignancy). The demographics of this group of patients are summarised in Table 5.7. Unfortunately there is no way of confirming whether the patients with available smoking history are representative of the whole group. However in the patients where the smoking history is available, the incidence of smoking was highest in patients with cervical cancer (54%; not taking into account the single patient with a Bartholin's gland tumour in view of the rarity of this tumour site).

Of the patients with a known smoking history a thrombotic event was diagnosed in 5 (3.55%) non-smokers, 3 (3.7%) smokers and 3 (5.88%) ex-smokers. It is difficult to comment on this incidence of thrombotic events according to the smoking history with so many patients having missing information.

Diagnosis	**Number of patients with available smoking history n=272**		
	Non-smokers n=141	**Smokers n=81**	**Ex-smokers n=51**
Cervical ca	23 (34%)	36 (54%)	8 (12%)
Endometrial ca	72 (65%)	15 (13%)	24 (22%)
Ovarian ca	29 (51%)	13 (23%)	15 (26%)
Vaginal ca	2 (40%)	2 (40%)	1 (20%)
Vulval ca	13 (45%)	14 (48%)	2 (7%)
Bartholin gland ca	0	1 (100%)	0
Unknown primary	1 (50%)	0	1 (50%)

Table 5.7: Summary of patient demographics according to smoking history.
Cancer (ca)

5.3.8 Disease stage

Information about the extent of disease (stage) was available in 73% (402 out of 553 patients; patients with pre-malignant conditions are not included in this analysis) of patients. There was an increased incidence of thrombotic events ($p<0.001$) and a worse prognosis (increased mortality rate; $p<0.001$) with more extensive disease stage. This data is summarised in Table 5.8.

Variable compared	Number of patients with available information re disease stage n=402			
	Stage 1 (n=209)	Stage 2 (n=71)	Stage 3 (n=61)	Stage 4 (n=61)
Total number of VTE events	5 (2.4%)	2 (2.8%)	8 (13.1%)	11 (18%)
Total number of arterial events	3 (1.4%)	2 (2.8%)	2 (3.3%)	0
Mortality rate	3 (1.4%)	5 (7%)	8 (13%)	22 (18%)

Table 5.8: Summary of the incidence of thrombotic events and mortality rate according to disease stage. Patients with pre-malignant conditions (CIN, VIN, VAIN and borderline ovarian tumours) have been excluded. Venous thromboembolism (VTE) refers to deep vein thrombosis and pulmonary embolism, arterial events refer to myocardial infarctions and cerebrovascular accidents.

5.4 DISCUSSION

I have collected data on all patients presenting with gynaecological malignancy in the South East of Scotland Cancer Network (SCAN) over one calendar year. All patients with a probable or confirmed diagnosis of gynaecological malignancy in SCAN are discussed at the gynae-oncology multidisciplinary meeting on a weekly basis. The patients' details, including their diagnosis and management plan, are recorded electronically onto the Lothian NHS IT system and were thus available to me for the purposes of the audit. I am therefore confident that my list of patients with a diagnosis of gynaecological malignancy is complete and truly representative of the gynae-oncology activity in SCAN.

All patients with gynaecological malignancy in SCAN are treated by the same team of gynae-oncologists based in Lothian. However the geographical area of SCAN is large (includes Lothian, Dumfries and Galloway, Fife and The Borders), so that while the management of the gynaecological malignancies is uniform across the whole region, acute complications such as VTE events are managed at the patients' local hospitals. Such hospitals outside Lothian are not linked up electronically to the Lothian NHS IT system, so that my data gathering of such acute events may be incomplete. I was dependent on timely correspondence being sent to the oncology team taking care of the patients' malignancy for information on the occurrence of such acute events. In view of the potential of having such information missing I analysed the data I gathered both as a whole group and also compared the two subgroups of patients according to the geographical area – group A being patients living within Lothian, and group B patients living outside Lothian but still within SCAN. In the case of patients in group A I am confident that I had full access to all their medical records through the Lothian NHS IT system and thus complete information as to the incidence of thrombotic events. When I compared the two subgroups of patients the incidence of the different types of gynaecological malignancies, thrombotic events and the mortality rate were very similar between the two subgroups, so that I am confident that my data gathering is in fact robust and truly reflects the patient population with gynaecological malignancy throughout SCAN – please refer to Table 5.1, page 135 and Table 5.4, page 145-146.

5.4.1 Incidence of gynaecological malignancies

The population living in SCAN is made up of approximately 1.45 million people, 0.7 million of whom are women (General Register Office for Scotland mid-2009 population estimate)[3]. During 2009 four hundred and seventy three women (excluding patients with pre-malignant conditions and with relapsed disease) presented with a new diagnosis of gynaecological malignancy, making the crude rate incidence for all types of gynaecological malignancies in SCAN 67.6 per 100,000 women. The details of the incidence for the different types of gynaecological malignancies are summarised and compared with the incidence in England in Table 5.9, page 161. There was a higher incidence of cervical, endometrial, ovarian and vulval cancer in SCAN when compared to England, although this difference did not reach statistical significance (p=0.317). These differences in the incidence rate may reflect different population demographics and risk factors for gynaecological malignancies in SCAN – for example smoking and obesity are two well established[229] risk factors for gynaecological malignancy. It would be useful to assess whether there are any significant differences in the population demographics and the population risk factors between SCAN and the rest of the UK that could explain the trend in the incidence rates of the gynaecological cancers.

The histological subtypes of the different gynaecological malignancies are described in Table 5.2, page 136-138. The distribution of the different histological subtypes in SCAN is similar to that reported in the published literature[230].

5.4.2 Incidence of thrombotic events

10.3% of all patients presenting with a gynaecological malignancy in 2009 in SCAN were diagnosed with a thrombotic event (8% VTEs, 2.3% arterial thromboses (CVA/MI)). The results are summarised in Table 5.4, page 145-146. The highest incidence of thrombosis was in patients with vaginal cancer: 40% of 5 patients. However the total number of patients diagnosed with vaginal cancer (5 patients) is very small so that it may be difficult to extrapolate these results to a bigger population of patients with vaginal cancer. Of the commoner gynaecological malignancies the patients with the highest incidence of thromboses were patients with ovarian cancer: 19.9% of 191 patients (14.7% VTE and 5.3% arterial events),

followed by patients with vulval cancer, 8.3% of 48 patients (6.3% VTE and 2.1% arterial events), patients with cervical cancer, 4.5% of 89 patients (3.4% VTE and 1.1% arterial events) and finally patients with endometrial cancer, 4.2% of 215 patients (3.7% VTE and 0.47% arterial events). This suggests that ovarian cancer tends to be more prothrombotic than other gynaecological malignancies.

There is very little in the way of prospective data in the literature assessing the incidence of thrombotic events in patients with gynaecological malignancy. The quoted incidence rates of VTEs are very variable, potentially reflecting the difficulty of collecting such data: 13.6-27% of patients with ovarian cancer[23], 9.8-57% of patients with endometrial cancer[8, 24, 25] and 0-34% of patients with cervical cancer[9]. Arterial thromboembolism occurs much less frequently than venous thrombosis in patients with malignancy[10, 231]. The incidence of thrombosis in the cohort of patients with gynaecological malignancy in SCAN is not dissimilar to the incidence rates published in the literature.

5.4.3 Patients not fit for treatment

In the SCAN patient cohort 30% of the thirty patients not eligible for treatment (either due to extensive disease, and/or patient co-morbidities) were diagnosed with a thrombotic event – Table 5.5, page 148. This thrombotic rate is likely to reflect the prothrombotic state of such patients as a result of the extent of disease, patient co-morbidities (e.g. renal disease, obesity) or concurrent illness (e.g. sepsis)[223, 224, 232].

5.4.4 Treatment

Oncology treatment can also contribute to the pro-thrombotic state of patients with cancer. The treatment offered to different patients varies, depending on the specific site of origin and histological subtypes, disease stage, any prior oncology treatment and patient fitness. Treatment consists of surgery, chemotherapy or radiotherapy (both external beam radiotherapy and brachytherapy), or a combination of such treatment modalities[4].

5.4.4.1 Chemotherapy

Cancer patients being treated with chemotherapy have been found to have a two- to six-fold increased risk of VTE compared to the general population[43, 223, 233]. There is also an increased risk of arterial events (CVA and MI) in such patients[231, 234]. In the SCAN patient cohort, patients being treated with chemotherapy had a significant incidence of thrombotic events: 6.5% for all chemotherapy regimens; 9.8% of patients treated with the combination of Carboplatin and Paclitaxel and 10% of patients treated with Carboplatin alone were diagnosed with a thrombotic event – Table 5.5, page 148. None of the 54 patients treated with a Cisplatin regimen were diagnosed with thrombosis while on treatment.

This incidence rate of thrombotic events is likely to reflect both the presence of the underlying disease as well as the effect of treatment with chemotherapy. Specific chemotherapeutic agents may be associated with higher rates of thrombosis than others. Platinum-based regimens have been significantly associated with thrombosis[223-225]. Cisplatin has been associated with platelet activation *in vitro*, elevated von Willebrand factor levels and hypomagnesaemia-induced vasospasm, which have been proposed as potential mechanisms for thrombosis[20, 235]. There are a number of publications linking Cisplatin treatment with thrombotic events, both venous and arterial[236]. All platinums have in fact been associated with an increased incidence of thrombosis, although most of the data available relates to Cisplatin. Paclitaxel has also been linked to an increased risk of thrombosis; it enhances the thrombin-induced TF expression in endothelial cells in a concentration and time dependent manner[226, 227]. It has been suggested that chronomodulation could result in a reduced incidence of thrombosis[223, 237].

Further studies are required to assess the risk:benefit ratio of primary thromboprophylaxis in patients being treated with chemotherapeutic agents that are known to be associated with an increased risk of thrombosis (such as platinums), especially because such patients also have a significant risk of bleeding.

5.4.4.2 Surgery

Surgery is a well known high risk setting for thrombotic events as a result of the trauma of surgery and immobility after surgery amongst other reasons. This is especially so in the presence of gynaecological cancer[17]. The presence of undiagnosed clinically silent DVT prior to surgery which extends and becomes symptomatic postoperatively may partly explain the high incidence of VTE events diagnosed postoperatively in this patient group. Thromboprophylaxis with TED stockings and low molecular weight heparin in the perioperative period has been shown to reduce the incidence of VTE events by approximately 15%[17, 38, 238-240], which in turn translates into a reduced mortality rate in the perioperative setting in patients with cancer[17]. Such thromboprophylaxis is recommended in all patients undergoing surgery for gynaecological malignancy.

In the SCAN audit cohort, of all the patients who required surgery as part of their management, only 4 (1.1% of all patients having surgery) were diagnosed with a thrombotic event (1VTE, 2MI, 1CVA) within 3 months of surgery. Of these 4 patients only one (0.3% of all patients having surgery) had a venous thrombosis – Table 5.5, page 148. This is a very low incidence of perioperative thrombotic events and is likely to reflect the routine use of thromboprophylaxis as described above in all patients having surgery for gynaecological malignancy, unless contraindicated.

5.4.4.3 Radiotherapy

Only one patient (0.6% of all patients receiving radiotherapy) was diagnosed with a VTE event while being treated with radiotherapy – Table 5.5, page 148. This patient had very extensive disease and radiotherapy was being used in a palliative setting to control her symptoms (pain and bleeding), making it likely that her disease extent was a more important contributing factor towards her thrombotic event.

Radiotherapy is generally not considered to be a risk factor for thrombosis, although there are only a small number of studies looking at this[223, 233, 241]. Treatment with radiotherapy can be associated with a number of risk factors for thrombosis, including potential dehydration secondary to torrential diarrhoea, immobility either as a result of treatment with brachytherapy or secondary to excessive fatigue and a

postulated prothrombotic effect on the endothelium. More studies are warranted to assess the thrombotic rate associated with radiotherapy treatment. The low incidence rate of thrombosis associated with radiotherapy in the audit cohort is likely to reflect the aggressive management of radiotherapy induced toxicities and the use of primary thromboprophylaxis in brachytherapy patients in SCAN.

5.4.4.4 Smoking

Unfortunately the data I have regarding the patients' smoking history is incomplete. It is unclear whether the group of patients with a documented smoking history is representative of the whole group of patients or not. However from the data available the incidence of smoking (54%) was highest in patients with cervical cancer. There is a postulated causal effect between smoking and cervical cancer. Smoking appears to enhance the progression of in-situ carcinomas to invasive carcinomas, and smokers tend to have a poorer response to radiotherapy and an increased incidence of radiotherapy associated toxicity[242].

There was no significant difference in the incidence of thrombotic events according to the smoking history (please refer to Table 5.7, page 152). However it is unclear whether this would still be the case if the data regarding the patients' smoking history was more complete.

5.4.5 Disease stage

There was an increased incidence of thrombotic events ($p<0.001$) and an increased mortality rate ($p<0.001$) in the audit cohort with more advanced disease – Table 5.8, page153. Large cohort studies have identified disease stage as a major risk factor for thrombosis[223, 243-245]. Advanced stage disease is associated with poor performance, and is more likely to be associated with disease related complications so that such patients are more likely to be unfit for treatment, or if eligible for treatment are more susceptible to treatment related complications. This translates into an increased mortality rate as the disease advances, a measure of the aggressiveness of the underlying malignancy. For example Stage I cervical cancer is associated with a 90% 5 year survival compared to <10% 5 year survival for Stage IV[246].

5.4.6 Mortality

The overall mortality rate during the observation period (median follow up of 11 months, range of 5-17 months) was 14.1%. While there was a trend for patients found to have a VTE event to have a worse outcome compared to patients with no thrombosis, the difference was not statistically significant.

Other studies have reported a significantly worse prognosis for cancer patients who were also found to have a VTE[6, 12, 19, 20], especially so if the latter occurred at the same time as the malignancy was diagnosed. The relationship between cancer and arterial thrombosis is less clear, with very little data in the published literature; Li et al.[231] reported very short survival times after ischaemic strokes in cancer patients being treated with chemotherapy.

There are some studies which have shown that heparin treatment may improve the prognosis of malignancy[58]. However this has not been confirmed by all studies and it is currently unclear what the role of heparin should be in patients with malignancy but no thrombosis, especially considering the risk of bleeding in such patients. It is currently unclear whether the worse prognosis associated with thrombotic events in patients with cancer is due to the thrombosis itself, or whether it reflects the presence of a more aggressive malignancy, and hence the worse prognosis.

Gynaecological malignancy	Number of new diagnoses in SCAN (2009)	Crude rate per 100,000 women in SCAN (2009)	Crude rate per 100,000 women in England (2007)
Endometrial cancer	199.0	28.4	24.0
Ovarian cancer	160.0	22.9	20.3
Cervical cancer	75.0	10.7	8.8
Vulval cancer	35.0	5.0	3.6
Vaginal cancer	2.0	0.3	0.8
Unknown primary	1.0	0.14	not available
Bartholin's gland cancer	1.0	0.14	not available
All types	473.0	67.6	not available

Table 5.9: Comparison of the incidence rate of the different gynaecological malignancies between SCAN and England. The total population living in SCAN is approximately 1.45 million, 0.7 million of whom are women. Patients with pre-malignant conditions (CIN, VIN, VAIN and borderline ovarian tumours) and patients with relapsed disease are excluded. Data for England was obtained from the Cancer Research UK website, June 2010[3]. The difference in incidence rates did not reach statistical significance (p=0.317).

5.5 COMPARISON OF MP STUDY COHORT AND SCAN AUDIT COHORT

I have compared the demographics of the patients recruited to the MP study with those of the SCAN audit cohort to establish whether the group of patients recruited to the MP study is representative of the whole population of patients with gynaecological malignancy treated in SCAN in 2009. The data is summarised in Table 5.10, page 163-164.

The relative proportions of the different types of gynaecological malignancy were very similar in the MP study cohort and the audit cohort (p=0.275, Mann Whitney *U* test). The incidence of venous thrombotic events (7.5% in MP study, 8% in SCAN audit, p=0.352, Mann Whitney *U* test) and the mortality rate (6% in MP study, 15.2% in SCAN audit, p=0.099, Mann Whitney *U* test) were also comparable. This suggests that the group of women in the MP study is representative of the overall population of women with gynaecological malignancy in SCAN.

It is not possible to comment on treatment related thrombosis in the patients in the MP study. Of the 5 patients diagnosed with a thrombotic event in this study, 4 presented with a thrombosis at the time their malignancy was first diagnosed, and only one patient had a thrombotic event while on chemotherapy.

Gynaecological malignancy	**Proportion of pts in MP study cohort (% of 67 pts)**	***Proportion of pts in SCAN audit cohort (% of 486 pts)***	**p value**
Endometrial cancer	36	*39*	n/s
Ovarian cancer	36	*34*	n/s
Cervical cancer	19	*16*	n/s
Vulval cancer	7.5	*9*	n/s
Vaginal cancer	1.5	*0.8*	n/s
Unknown primary	0	*0.6*	n/s
Peritoneal mesothelioma	0	*0.2*	n/s
Bartholin's gland cancer	0	*0.2*	n/s

Table 5.10a: The proportion of patients diagnosed with the different gynaecological malignancies in the microparticle (MP) study cohort compared to the cohort of patients in the SCAN audit.

Gynaecological malignancy	**Proportion of pts with VTE on ca Dx (%)**		**Proportion of pts with VTE post ca Dx (%)**		**Total (VTE pre & post ca Dx) (%)**		**p value**
	Study cohort	*Audit cohort*	Study cohort	*Audit cohort*	Study cohort	*Audit cohort*	
Endometrial ca	0	*2.6*	0	*1.6*	0	*4.2*	n/s
Ovarian cancer	8.3	*10.2*	4.2	*4.8*	12.5	*15*	n/s
Cervical cancer	0	*1.3*	0	*2.6*	0	*3.9*	n/s
Vulval cancer	40	*0*	0	*2.3*	40	*2.3*	n/s
Vaginal cancer	0	*25*	0	*25*	0	*50*	n/s
All types	6	*4.9*	1.5	*3.1*	7.5	*8*	n/s

Table 5.10b: Comparison of the proportion of patients with a venous thromboembolic (VTE) event in the MP study cohort and the audit cohort.

	Study cohort	*Audit cohort*	p value
Proportion of patients who died (%)	6	*15.2*	n/s
Proportion of patients with a venous thrombotic event who died (%)	40	*59*	n/s

Table 5.10c: Comparison of the proportion of patients who died by June 2010 (median follow up of 11 months, range 5-17 months).

The 67 patients in the MP study have been removed from the audit cohort (n=584) for the purposes of the above comparisons; patients with pre-malignant conditions (n=31) have also been excluded. This means that for the above comparisons n=486 (584 minus 67 minus 31) for the audit cohort. There is no significant difference in the above relative proportions, so that the cohort of patients in the MP study is representative of the overall population of patients with gynaecological malignancy in SCAN.
Cancer (ca), patients (pts), diagnosis (Dx), not statistically significant (n/s). The p value for 2-tailed significance testing has been calculated using the Mann Whitney *U* test for 2 independent samples.

5.6 CONCLUSION

From the SCAN audit data I have found that the incidence rate of thrombosis in the patient cohort diagnosed with gynaecological malignancy in SCAN was 10.3% (8% VTE and 2.3% arterial events) in 2009. Of the commoner subtypes of gynaecological malignancy, patients with ovarian cancer were most at risk of a thrombotic event. The highest incidence of thrombosis was in patients too unfit for treatment (30%). Patients being treated with chemotherapy also had a significant incidence (6.5%) of thrombosis, likely reflecting the prothrombotic nature both of the underlying disease, and also treatment with chemotherapy agents. There was no significant increase in thrombosis associated with radiotherapy (0.6%) or surgery (1.1%). In the latter case the low rate of thrombosis is likely to be a reflection of routine primary thromboprophylaxis in the perioperative period in patients having surgery for gynaecological malignancy.

I have compared the group of patients recruited to the MP study with the SCAN audit cohort and found that the MP study cohort is representative of the SCAN audit cohort. This suggests that the results of the study on MPs can be extrapolated to the whole population of women diagnosed with gynaecological malignancy in SCAN.

CHAPTER VI

GENERAL DISCUSSION

The aim of this research project was to test the hypothesis that the number of MPs is raised in patients with gynaecological malignancy with thrombosis and that they are a useful marker of thrombotic risk. I also wanted to compare the number of plasma MPs in patients with gynaecological malignancy and a control group of women without malignancy. This required the establishment of a robust, reproducible and practical method to measure plasma MPs. I also wanted to establish whether there was any correlation between the number of plasma MPs and established markers of haemostatic activation such as D-dimers.

6.1 Measurement of plasma microparticles

Despite a plethora of published articles in the peer reviewed literature indicating that MPs are likely to be important contributors in many different patho-physiological processes, there is still no universally accepted definition of what constitutes a MP, nor any standardised method of MP identification and measurement.

MPs are generally considered to be anucleate structures, 0.1-1μm in diameter, surrounded by a phospholipid bilayer, released by microvesiculation from the plasma membrane of cells[73, 76, 79]. They contain various different cytoplasmic components and also express surface antigens, proteins and receptors of their parent cell. They circulate throughout the body and transfer receptors, genetic information (mRNA) and second messengers to recipient cells, allowing intercellular communication between far removed cells. They are also able to support haemostasis on their surface phospholipids.

MPs are released from cells either secondary to cell activation or cell stress/apoptosis. The release of MPs is a physiological process. During cell activation or apoptosis there is an increase in intracytoplasmic calcium which leads to the activation of various different enzymes (please refer to Figure 1.1, page 11) resulting in membrane budding and MP shedding by microvesicualtion. Blood cells can continue to release MPs after blood sampling. It is well recognised that variable pre-analytical handling of samples can significantly affect the number of MPs detected. These pre-analytical variables have not yet been fully characterised.

It is important to avoid cell/platelet activation/stimulation during blood sampling. The use of minimal or no stasis when sampling blood and the use of at least a 21 gauge needle is therefore recommended[78]. Blood sampled into anticoagulants that preserve extracellular calcium e.g. heparin, PPACK allow the continuing release of MPs post blood sampling[78]. This happens to a lesser degree when blood is sampled into anticoagulants that chelate calcium e.g. citrate, ACD. In the majority of published studies MPs have been measured from samples taken into citrate tubes[74, 81, 82, 116, 142]. Given that MP release *in vitro* can happen, samples need to be handled in as uniform a manner as possible to ensure that results are comparable. In this study all samples were handled by one operator. Blood was withdrawn with minimal or no venous stasis using a 21 gauge needle, and collected into citrate tubes to minimise the *in vitro* release of MPs.

The interval between blood sampling and MP analysis is also likely to contribute to variable results due to the potential for continuing *in vitro* release of MPs. Many investigators[81, 116, 142, 159] do not specify the length of time between blood sampling and either freezing the samples or analysing MPs in fresh plasma. Variable lengths of storage times are likely to contribute significantly to the variability of results in the published literature. I addressed this by ensuring that the method used was tightly controlled and that all samples were analysed within two hours of blood sampling.

It is also unclear what effect a freeze-thaw cycle has on the number of MPs, with different investigators reporting different and sometimes opposite effects. For example Rectenwald[35] reported increased number of MPs when the PFP had been stored frozen prior to analysis, while Shah[78] found that MP numbers decreased after storage irrespective of whether the PFP was stored at room temperature or frozen. The majority of published studies have analysed MPs on samples stored frozen [35, 81, 82, 116, 140, 142, 159, 164] and then thawed by a variety of different methods prior to analysis, with few studies reporting on MP numbers in fresh plasma[212, 213]. This makes the meaningful comparison of results published in the literature difficult and is likely to be contributing to significant variation in the results published. Limiting MP analysis to fresh samples significantly limits the usefulness

of the assay, prevents sample batching and makes inter-laboratory collaboration and comparison difficult. However it is clear from the published results that a freeze-thaw cycle has an as yet undefined effect on MPs. At the start of patient recruitment I compared the number of MPs identified in fresh plasma and a frozen aliquot of the same plasma. The results obtained from plasma that had gone through a freeze/thaw cycle did not correlate with the results from fresh plasma (please refer to section 3.5, page 64). In view of this I measured MPs on fresh plasma to avoid any effect a freeze-thaw cycle may have on MP numbers. Further research to clarify the effect a freeze-thaw cycle has on MP numbers is important since the ability to carry out MP analysis on plasma that has been stored frozen would greatly enhance the clinical usefulness of this assay.

It is equally important that the *in vitro* release and loss of MPs secondary to centrifugation or washing is minimized. The centrifugation protocols used vary significantly between different laboratories (please refer to Table 2.1, page 40). This is likely to be contributing to variable published results. The speed and duration of centrifugation may lead to either the potential "generation" of MPs, when plasma is ultra-centrifuged, or to the loss of MPs which are not sedimented out. If washing is also used this may further contribute to MP loss. There is no single centrifugation protocol that will guarantee that all MPs are isolated, particularly as some MPs are no denser than plasma. I prepared PFP using serial centrifugation. I avoided the use of ultracentrifugation due to the concern of potentially "generating" MPs during such high speed centrifugation. After a literature review I compared 3 different serial centrifugation protocols to establish which protocol would allow me to prepare PFP with a residual platelet count consistently less than $5x10^9$/L. I confirmed the residual platelet count in all the PFP samples and in 93.4% of the samples there were no or $<5x10^9$/L residual platelets. Seven (6.4%) of the samples had a residual platelet count of 6-$9x10^9$/L. I found a statistically significant correlation between the number of residual platelets in the plasma and the number of PMPs measured after centrifugation (please refer to section 4.4.7, page 101). It is likely that some of the residual small platelets were being measured with the PMPs. Most investigators[35, 74, 81] do not quantify the number of residual platelets in the PFP. This is likely to partly explain the variable results from different investigators using different

centrifugation protocols, particularly as the number of residual contaminating platelets will vary according to the centrifugation protocol used, therefore having a variable effect on the number of MPs detected. So in the absence of any standardised protocol, I have selected procedures to minimise *in vitro* generation or loss of MPs, and have used a consistent approach to allow intra-study comparability.

A further source of variable results is the specificity of antibodies used to identify different MP subtypes. Initially MPs were thought to universally bind AV which could therefore be used as a marker for all subtypes of MPs, irrespective of their cell of origin. There are now a number of published articles[73, 87] indicating that AV negative MPs can make up a significant, and in some cases the majority, proportion of all plasma MPs. Unfortunately there is again variability between investigators as to whether AV positivity is used as a defining criteria for MPs or not. For example Hron et al[81] and Toth et al[164] only analysed AV positive MPs, while Helley et al[82] and Rectenwald et al[35] analysed all MPs, irrespective of AV positivity. In view of the fact that it is now clear that AV negative MPs may make up a significant proportion of plasma MPs, using AV positivity to define MPs could potentially result in a significant and variable proportion of plasma MPs being not identified, again making the meaningful comparison of results between different investigators difficult. In my study AV+ve MPs made up a minority of all MPs detected, so that measuring only AV+ve events would have meant that the majority of MPs would have been missed (please refer to section 4.4.4, page 96). When comparing results in the literature, this needs to be taken into account.

The choice of cell-specific antibody to identify different subtypes of MPs also contributes to variable results. The specific antigens and the concentration at which they are expressed by MPs vary according to the stimulus that leads to the release of MPs. For example CD41+ve PMP and CD42+ve PMP populations are not identical and reflect different pathophysiological phenomena. Depending on which cell-specific antibody (e.g. anti-CD41, anti-CD41a, anti-CD42, anti-CD61) is used to identify PMPs, different subgroups of PMPs are measured, leading to different results. For example Toth et al[164] used CD61 to identify PMPs, while Hron et al[81] identified PMPs using CD41a. Unfortunately there is currently no universal

marker for MPs irrespective of cell of origin and no marker that will identify all MPs released from a particular cell type irrespective of the stimulus that lead to their release. This will contribute to different results published by different investigators. Standardising the specificity of antibodies used would facilitate the meaningful comparison of results between different laboratories. The antibodies I have used to identify the cell-specific MPs were identified after a literature review and chosen due to their specificity for the particular cell types (please refer to section 3.3.4, page 54-56). The choice of antibodies used needs to be taken into consideration when comparing results from different investigators.

When I initially designed the study I planned to use combinations of antibodies (using multicolour flow) in order to be able to identify the cell of origin of AV+ve MPs and TF+ve MPs. In my initial experiments I realised that the binding buffer necessary for AV binding was interfering with the binding of the cell-specific antibodies. Likewise anti-CD142 was also interfering with other antibodies. I therefore elected to use AV and anti-CD142 singly, so that while I was able to measure the total number of AV+ve MPs and the total number of TF+ve (CD142) MPs, I have been unable to identify their cell of origin. It would be valuable to be able to characterise such MPs further. It would be particularly interesting to establish the cell of origin of TF+ve MPs since this may be of therapeutic significance; for example to establish whether the TF+ve MPs are originating from tumour cells or other cells. This could be studied further by using other antibody combinations so as to better characterise AV+ve and TF+ve MPs.

There are a number of different assays that can be used to analyse MPs. The technique used most often is flow cytometry. This has the advantage of analysing tens of thousands of MPs and gives information regarding the size and complexity of the MPs being analysed. It however gives no information as to the concentration of the antigen being measured on individual MPs or the functional properties of the MPs, unlike ELISAs and functional assays (see later).

When measuring MPs using flow cytometry, MPs are identified based on size and antigen positivity. MPs are most often defined as being 0.1-1μm in diameter[74, 81,

82]. The lower size limit was chosen due to the fact that smaller particles cannot readily be distinguished from electronic noise when using flow cytometry. Current flow cytometers were intended to measure cells much larger in size than MPs. Their small size means that most MPs appear close to the electronic noise of current flow cytometers. The accuracy of measuring such small particles with currently available flow cytometers is highly dependent on fine optical adjustments and fluidics and optics cleanliness of the machine. This will vary from one machine to the next and depends on maintenance for a single instrument. This was borne out in the results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop[247], where it was shown that standardization of PMP enumeration by flow cytometry is feasible, but is dependent on intrinsic characteristics of both the flow cytometer and the calibration strategy. It is now clear that most current flow cytometers are unable to accurately and specifically identify and measure particles less than 0.5μm in diameter. This means that a significant and variable proportion of the circulating plasma MPs will be missed by current flow cytometers.

The upper size limit of MPs is also arbitrary, mainly due to the fact that flow cytometers are unable to distinguish between PMPs larger than 1μm and platelets smaller than 1μm in diameter, both platelets and MPs being on a continuum of size. This invariably contributes to variable results. Using size as one of the defining criteria of MPs (as in my study) necessarily results in some MPs being missed and some small platelets being measured with the PMPs. At present there is however no easy solution to this problem.

There is currently a significant amount of research being carried out to try and improve the accuracy and specificity of MP identification, looking at the use of impedance based flow cytometers and digitally acquiring flow cytometers amongst other techniques. As the technology evolves and is better able to identify such small structures it may well be that MP assays will become much more accurate and easier to standardise. My method of analysing MPs was based on flow cytometry. I defined MPs as events <1μm in size that were positive for a particular cell specific antigen. To maximise the specific identification of MPs I set a lower threshold on forward scatter such that small MPs would not be missed while at the same time

removing some of the electronic noise of the machine. To avoid any variability in results due to technique all analyses were carried out on a single instrument using a constant setup everytime.

MPs may also be analysed using ELISAs or functional assays. When using such assays MPs are first isolated and immobilised from PFP using specific monoclonal antibodies or AV. In ELISAs a second monoclonal antibody is used to identify and quantify MP-bound antigens, while in functional assays, the functional properties of the MPs are assessed using a prothrombinase assay. In such assays the antibodies used will capture MPs irrespective of their size (unlike in flow cytometry). ELISAs and functional assays however still have the same problems as flow cytometry of potentially loosing/generating MPs according to the technique used to prepare PFP and similar problems regarding the specificity of the antibodies used to identify the MPs since there is currently no universal marker that will identify all MPs irrespective of cell of origin. It is now clear that the results from flow cytometric analysis of MPs are not interchangeable with the results obtained from ELISAs or functional assays. There are several publications[81, 116, 159] where the results of the various types of assays did not correlate. For example Haubold et al[159] reported significantly increased TF activity in cancer patients, but the TF antigen levels and TF+ve MP levels did not correlate with TF activity. It would be valuable to set up a functional based assay to assess the procoagulant activity of the MPs, and compare the results of such assays with the number of MPs measured by flow cytometry.

In order to quantify the absolute number of MPs in cell free plasma I used a flow based method using TRUCOUNT™ beads (please refer to section 3.4.2, page 61). A number of studies have reported that the flow rate through a flow cytometer is constant[221]. This was borne out in my study too. The volume of fluid analysed by the flow cytometer over 120 seconds was very stable over a session of analysis, and the overall inter-assay CV over the twelve month period of analysing patient samples was 3.2%. With this method TRUCOUNT™ beads are only required at the start and again at the end of each session of patient samples, making it a more cost-effective method than adding such or similar beads to each patient tube.

It is clear that while there is currently a tremendous amount of research being carried out and many peer-reviewed articles being published on MPs there are still many important unanswered questions, not least how best to analyse MPs to ensure reproducible results. It is very important that any laboratory carrying out research on MPs is meticulous in standardising their protocol used to analyse MPs. This would at least ensure that their results are comparable for their samples and would be comparable with another laboratory's results using the same protocol. Unfortunately the pre-analytical details are not always described in a such way as to accurately establish their effects on published data, but will contribute to the variability of published results. In order to ensure that my results from different patients are comparable I standardised the pre/analytical process. All samples were analysed by me using a tightly controlled protocol (please refer to chapter III) to ensure that any variability secondary to pre/analytical handling was kept to a minimum. This means that while my results may not be comparable with those published using different pre-analytical and analytical techniques, they are comparable between themselves.

6.2 Study results

As far as I can ascertain this is the first study measuring MPs in patients with gynaecological malignancy. I measured the number of plasma MPs in a cohort of women diagnosed with gynaecological malignancy and a control group of women without malignancy. Gynaecological malignancy was chosen due to the fact that gynaecological malignancies are associated with a significant incidence of thrombosis. Although the different gynaecological malignancies have diverse pathologies they are all managed by the same clinical team in Edinburgh, so that the data collected would be comprehensive and consistent.

The control group of women was on average younger than the malignant group. However this is unlikely to have affected the MP results, there being no correlation between age and MP numbers (please refer to section 4.3.1, page 82 and 4.4.5, page 97). Control groups in the published literature have varied. Hron et al[81] measured MPs in 20 patients with colorectal cancer and compared the results with 20 healthy control subjects matched for age and sex. On the other hand in the study by Rectenwald et al[35], the control group was significantly younger than the patient group with no analysis made as to whether MP numbers were correlated with age.

MPs have been measured in many different patient groups by other investigators. There are several reports[18, 164, 165] in the published literature indicating that the number of plasma MPs is increased in various different prothrombotic conditions including malignancy. There are however also studies where the number of MPs measured was similar between patients with cancer and a control group without cancer, but have at the same time reported an increased numbers of TF+ve MPs[81] or increased MP associated TF function[116]. For example Hron et al[81] reported increased numbers of TF+ve MPs in cancer patients compared to healthy controls, however the absolute number of MPs in plasma did not signficantly differ between patients and controls. The protocol used by Hron et al was significantly different to that used in my research study. PFP was prepared by a one-step centrifugation at 2600*g* for 15 minutes (*vs* 1500gx15minutes + 13,500gx2minutes); plasma samples were stored frozen rather than processed fresh; the MPs were defined by positivity to both AV and cell-specific antibodies thus exluding AV negative MPs (unlike in my

study, please refer to chapter III for study protocol). Tesselaar et al[248] reported that AV+ve MP numbers did not differ in patients with cancer with VTE compared to cancer patients without VTE, but found increased TF-dependent MP activity in the cancer patients with VTE. The protocol used in this study is again different. PFP was prepared with a one-step centrifugation at 1550*g* for 20 minutes (*vs* 1500gx15minutes + 13,500gx2minutes) and plasma samples were stored frozen rather than processed fresh. Kanazawa et al[249] on the other hand reported increased numbers of PMPs in lung cancer patients compared to healthy controls. In this latter study the centifugation protocol used to prepare PFP was different again, this time including washing steps; the plasma samples were analysed fresh (as in my study), but the PMPs were identified using different cell specific antibodies compared to my study. The studies described above are all significantly different in between themselves and different from my study protocol. There isn't one easily identifiable confounding variable that could readily explain the variable published results. It is more likely that a combination of pre/analytical variables contribute to the different results. It may also be that differences in the underlying pathologies are contributing to the variability in the published literature.

Although all the patients in my study had a diagnosis of gynaecological cancer, they are in fact a relatively diverse group due to the fact that different types of gynaecological cancer have a different prognosis and behave differently biologically. Other investigators have however reported a difference in the number of plasma MPs between a diverse group of cancer patients and control groups without cancer[18, 140, 144]. For example Tesselaar et al[116] reported increased AV+ve MPs in a group of patients with pancreatic (n=23) and breast (n=27) cancer compared to a group of healthy control (n=37), and Zwicker et al[18] reported increased TF+ve MPs in patients with pancreatic (n=39) and colorectal (n=12) cancer compared with cancer-free controls (n=31). There is increasing evidence that MPs are likely to contribute to cancer cell survival, invasiveness and metastases[99, 109]. Given this, it is reasonable to hypothesise that plasma MPs would be present in increased numbers in patients with cancer. The fact that I did not find a difference in plasma MPs between the malignant group and the control group should not have been influenced by the specific type of gynaecological cancer present, although it is

possible that a more uniform and bigger group of cancer patients may have allowed a difference in MP numbers to be identified. It is however also likely that the lack of a standardised method of analysis, particularly in the pre-analytical handling of samples, is contributing to the variable reports in the literature.

Given the fact that MPs express surface negatively charged phospholipids and also carry TF, other clotting factors, vWF and receptors for clotting factors[84, 147, 148], MPs are generally considered to be important components of normal haemostasis and to contribute to thrombosis when present in increased numbers. Indeed MPs have been reported to be 50-100x[146] more procoagulant than an identical surface of activated platelets. In view of this it would be reasonable to speculate that MPs can in fact contribute towards thrombosis.

In this study I wanted to establish whether measuring MP numbers in patients with gynaecological malignancy would be helpful in identifying patients at an increased risk of thrombosis. The statistical advice obtained prior to study recruitment suggested that assuming approximately 25% of women with gynaecological cancer would develop a VTE at some point during their treatment for cancer (as per quoted incidence rates in the literature[6, 8, 9, 20, 24, 27, 250]), a consecutive series of around 140 women with gynaecological cancer would have a 90% probability of detecting a difference in the mean number of MPs between patients with a VTE and patients with no VTE. We also aimed to recruit a similar number (n=140) of women without malignancy to explore whether MP analysis is a superior predictor of VTE in women with gynaecological cancer compared to women without cancer. Interim study analysis after 12 months of study recruitment indicated that the incidence of VTE in the study (7.5%) was significantly lower than expected so that the planned study was underpowered to identify a difference in MP numbers in patients with VTE compated to patients with no VTE. Furthermore the number of plasma MPs in the patients with VTE was indistinguishable from the number of MPs in patients without a VTE event. Neither was there any difference or trend in the number of plasma MPs in patients with gynaecological cancer as a group compared to the control group. It was therefore decided at that point to stop study recruitment since it was unlikely that there would be a statistically significant difference in MP numbers

between the identified patient groups. It is possible that a much larger study or longer follow up may have resulted in more of the study patients being diagnosed with a VTE, allowing any difference that may have been present in MP numbers to be identified.

Due to the fact that the number of patients diagnosed with a VTE event in the study was much smaller than initially expected, I audited all the patients diagnosed with gynaecological malignancy within SCAN (the geographical area from where the study patients were recruited) over one calendar year, with the aim of establishing the incidence of the various types of gynaecological malignancy in the overall population being treated in SCAN and the incidence of VTEs in this patient group.

All the patients treated for gynaecological malignancy in SCAN are managed by one team of gynae-oncologists. All such patients are discussed at a weekly multi-disciplinary meeting and their details are captured on a local IT system. This allowed me to identify all patients presenting with gynaecological malignancy within one calendar year. After analysing all the data (please refer to chapter V) it was clear that the group of patients recruited to my study was in fact representative of the overall population within SCAN. The incidence of VTE events was very similar in the overall population of patients diagnosed with gynaecological malignancy in SCAN (8%) to that of the study patients (7.5%). This suggests that the results of this study can be extrapolated to the SCAN population of patients diagnosed with gynaecological malignancy. The results of the SCAN audit confirmed that I would have needed to recruit a much larger number of patients than originally planned in order to detect any statistically significant difference in the number of MPs measured in cancer patients with a VTE compared to cancer patients without VTE. This SCAN data, previously unavailable, has been presented as an abstract at the 17th International Meeting of the European Society of Gynaecological Oncology.

I also measured indirect markers of haemostatic activation as a surrogate marker in the patients with cancer and the control group. Similar to other publications in the literature[171] the levels of D-dimers and PF1&2 were statistically significantly increased in the patients with gynaecological malignancy (even after adjusting for

age), evidence of haemostatic activation in patients with malignancy (please refer to sections 4.4.13-4.4.20, pages 110-120). The level of D-dimers was also statistically significantly increased in the small group of patients with a VTE event. The level of TATs was not statistically significantly different between the two groups. The latter is likely to reflect the lower sensitivity of TATs[50] and their shorter half life in circulation.

The different markers of haemostatic activation were statistically significantly correlated, however there was no correlation between the level of D-dimers, TATs and PF1&2 and the number of MPs measured. This is in keeping with the fact that there was no difference detectable in MP numbers measured between the two groups of patients. My results therefore do not support the hypothesis that the number of plasma MPs correlate with thrombosis.

6.3 Study limitations

Some of the limitations of the study have already been touched on in the preceeding discussion. This is a single institute study, with a relatively short follow up (median 11 months, range 5-17 months). Although all the patients recruited had a diagnosis of gynaecological cancer, the latter types of cancer have a varied biological course with different prognoses and outcomes depending on the specific type of gynaecological cancer present. This means that the patient group recruited was not uniform. However as already discussed, other studies (see previously) have previously reported a statistically significant difference in the number of MPs measured between cancer patients (variable diagnosis) and patients with no underlying cancer, in some cases with smaller numbers recruited than in this study.

Although the incidence of VTEs in the study cohort was similar to that of the overall population of patients presenting with gynaecological cancer in SCAN, the absolute number of such patients was too small to be able to form any definitive conclusions as to the usefulness of measuring MP numbers as a marker of thrombotic potential. A much larger study is needed in order to have enough patients diagnosed with a VTE event.

Due to logistical reasons patients were not recruited in a consecutive manner; however there was no perceived bias and when comparing the incidence of the various types of gynaecological cancer and the incidence of VTEs in the study group, this was comparable with the overall population of patients presenting with gynaecological cancer in SCAN (please refer to chapter V).

The MPs were measured using light scattering flow cytometry. While this is the most commonly used technique to measure MPs it is clear that the technology currently struggles to accurately and specifically measure MPs, particularly ones smaller than 0.5μm in diameter. I have not looked at the functional properties of the MPs measured; published studies[140, 248] indicate that such an assay may be more helpful in assessing the thrombotic potential of individual patients.

Due to difficulties with antibody interference I have been unable to identify the cell of origin of the TF+ve MPs and of the AV+ve MPs. This could be studied further by using other antibody combinations. It would be very helpful to be able to identify which cells are releasing the TF+ve MPs, particularly if the TF+ve MPs are originating from cancer cells.

6.4 Conclusion

I have set up an assay using light-scattering flow cytometry to measure plasma MPs. All samples were analysed using a tightly controlled protocol on fresh plasma. My results may not be readily comparable to other studies due to differences in methodology. Keeping in mind the limitations of the study, there was no difference in plasma MP numbers between patients with gynaecological malignancy and a control group of women without malignancy. The number of patients in the study diagnosed with a VTE is too small to draw any firm conclusions; however the lack of correlation with surrogate markers of haemostatic activation, and the current difficulty of accurately measuring the number of plasma MPs, would suggest that MPs are not a good clinical marker of thrombotic risk in this patient group.

APPENDIX A

Covering Invitation Letter

Date:

Dear Ms.

Following your consultation in hospital with Dr _____, I would like to invite you to take part in a research project we are carrying out. I would be very grateful if you were to read the attached information sheet giving you details of our research project and what your involvement would be should you decide to help us with this research project.

Thank you for your time and help.

Best regards,

SHARON ZAHRA
Clinical Lecturer in Haematology
University of Edinburgh
Department of Haematology
Royal Infirmary of Edinburgh
51 Little France Crescent
Edinburgh EH16 4AC

APPENDIX B

Patient information sheet – Gynaecological malignancy patients

MICROPARTICLES AND THROMBIN GENERATION IN PATIENTS WITH GYNAECOLOGICAL MALIGNANCY

I. Invitation

You are being invited to take part in a research study. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part in the study.

II. Introduction

You have recently been diagnosed with cancer. Patients vary widely in the incidence of side-effects from cancer and its treatment, in particular the incidence of blood clots. If a test could be developed to accurately predict the patients at high risk of developing blood clots then those patients may in the future be offered medication to try and reduce this risk, while patients thought to be at low risk of developing a blood clot could be spared unnecessary medication.

III. Standard Management

As part of your normal management, you will be offered treatment with one or more of surgery, chemotherapy, radiotherapy and brachytherapy depending on the exact nature of your cancer. During your treatment your doctors will be taking blood samples at regular intervals to monitor your response to the treatment you are receiving.

IV. Participation in the Study

This study is aimed at establishing what happens to the level of microparticles (very small particles in the blood) and to thrombin generation (the rate of production of a protein in the blood) in patients with gynaecological cancer, and whether these change during the course of the disease and its treatment. We will also be looking to see whether we can establish a link between the level of microparticles, thrombin generation and the onset of blood clots. This will be done by analysing blood samples from patients. We hope to enrol 140 patients in the study over 2 years.

Microparticles are small particles that are found circulating in everybody's blood. They are known to be involved in inflammation and blood clotting, and while they are present in healthy individuals, their levels tend to increase significantly in many disease states, including cancer. Microparticles may therefore represent novel markers of disease activity. They may also be involved in several disease processes such as the development of blood clots.

Thrombin generation is a relatively new technique that allows us to measure an individual's ability to stop bleeding or to develop blood clots.

- By taking part in this study you will get exactly the same treatment as you would otherwise.
- We will ask you for a small blood sample (12mls – approximately the size of a table spoon) over and above the blood samples that will be taken as part of your normal management, for us to analyse in the laboratory
- The number of blood samples that we would need will vary according to the treatment you will receive. Depending on which type of cancer you have you may be treated with any or all of surgery, chemotherapy, radiotherapy and brachytherapy – depending on your treatment we would like to take blood samples just before and after surgery, at regular intervals during chemotherapy and radiotherapy, before and after brachytherapy, and should you develop a blood clot. In view of this the total number of blood samples that we would need from you will vary from just two samples up to a maximum of ten samples.

- We will endeavour to time our blood samples so that they are done at the same time as the blood samples that you will need to have done as part of your usual management. You will be free to stop having blood samples taken for our study at any time.
- The blood samples that we will take will be stored in our laboratory and analysed as necessary to establish the level of microparticles and thrombin generation, and other markers of activation of the clotting mechanism. The blood samples will be disposed of at the end of our research project.
- We would like to contact you for a further blood sample should you require further treatment for your cancer.
- We would like to send a letter to your GP to inform him/her of your participation.
- If you have private medical insurance, you should contact your insurance company to ensure that your participation will not affect your insurance in any way.
- Participation in the study is entirely voluntary. By agreeing to take part in the study you will remain completely free to opt out at any stage, and to withdraw your consent to any part of the process, without having to give a reason. The decision not to participate or to withdraw from the study will not affect the standard of care you receive.
- If you choose not to participate in the study your treatment will be unaffected and will go ahead entirely as normal.
- Please let us know if you are participating in any other research projects.

V. What happens if something goes wrong?

If you are harmed by taking part in this research study, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

VI. Confidentiality

If you consent to take part in this study, your medical records will be inspected by the investigators conducting this study. All information that is collected about you will be kept strictly confidential. Any information leaving the hospital will have your name and address removed so that you cannot be recognised.

VII. Funding of the Study

This study is supported by funds through the University of Edinburgh.

VIII. Results of the Study

The initial results of the research will be available about 2 years from the start of the study and will be published in peer reviewed medical journals. We do not expect our findings to alter the management of current patients; however it is hoped that they might reveal important correlations between blood clots and the level of microparticles, which may be of clinical relevance to patients in the future. We would thus like to be able to use the results we obtain from this study to compare with the results from other potential future studies. We will be happy to let you know our overall results at the completion of our study. If you would like to be informed in writing please let us know.

IX. New Information

If any new information becomes available during the course of this study, which might affect your willingness to continue taking part, your doctor will inform you immediately.

X. Contact for further information

If you require further information about this study, please contact Dr. Sharon Zahra at the New Royal Infirmary of Edinburgh on 0131 242 6096.

Thank you for taking the time to read this information sheet. Your participation in the study would be greatly appreciated and highly valued, as it would allow us to further our knowledge and help us treat future patients.

APPENDIX C

Patient information sheet – Patients with benign gynaecological conditions requiring surgical treatment

MICROPARTICLES AND THROMBIN GENERATION

I. Invitation

You are being invited to take part in a research study. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part in the study.

II. Introduction

You have been chosen because you are about to undergo surgery for a gynaecological condition. We need to recruit a group of people like yourself as controls for a study we are carrying out in patients with various different gynaecological conditions.

We are looking at the level of microparticles and thrombin generation. We need to compare these results in different patient groups, and need to recruit a group of patients about to undergo surgery for a benign gynaecological condition such as yourself as one of our control groups.

III. Standard Management

As part of your normal management, you will be offered gynaecological surgery, and your doctors will monitor your blood tests.

IV. Participation in the Study

Our aim is to compare the level of microparticles and thrombin generation in patients requiring treatment for various different gynaecological conditions. We will be looking to see whether we can establish a link between the level of microparticles, thrombin generation and the onset of blood clots. This will be done by analysing blood samples from patients. We hope to enrol a group of 140 patients similar to yourself into the study over a 2 year period.

Microparticles are small particles that are found circulating in everybody's blood. They are known to be involved in inflammation and blood clotting, and while they are present in healthy individuals, their levels tend to increase significantly in many disease states. Microparticles may therefore represent novel markers of disease activity. They may also be involved in several disease processes such as the development of blood clots.

Thrombin generation is a relatively new technique that allows us to measure an individual's ability to stop bleeding or to develop blood clots.

- By taking part in this study you will get exactly the same treatment as you would otherwise.
- We will ask you for a small blood sample (12mls – approximately the size of a table spoon) over and above the blood samples that will be taken as part of your normal management, for us to analyse in the laboratory.
- The blood samples that we need will be taken just before and after your surgery. We will also ask you for a further blood sample should you develop a blood clot around the time of surgery or in the subsequent three months.
- The total number of blood samples that we would require would thus be two in the majority of cases, or a total of five should you develop a blood clot.
- We will endeavour to time our blood samples so that they are done at the same time as the blood tests that you will need to have done as part of your usual management.

- We would like to contact you by telephone at one month and three months after your surgery to check on your progress.
- The blood samples that we will take will be stored in our laboratory and analysed as necessary to establish the level of microparticles and thrombin generation, and other markers of activation of the clotting mechanism. The blood samples will be disposed of at the end of our research project.
- We would like to send a letter to your GP to inform him/her of your participation.
- If you have private medical insurance, you should contact your insurance company to ensure that your participation will not affect your insurance in any way.
- Participation in the study is entirely voluntary. By agreeing to take part in the study you will remain completely free to opt out at any stage, and to withdraw your consent to any part of the process, without having to give a reason. The decision not to participate or to withdraw from the study will not affect the standard of care you receive.
- If you choose not to participate in the study your treatment will be unaffected and will go ahead entirely as normal.
- Please let us know if you are participating in any other research projects.

V. What happens if something goes wrong?

If you are harmed by taking part in this research study, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you.

VI. Confidentiality

If you consent to take part in this study, your medical records will be inspected by the investigators conducting this study. All information that is collected about you will be kept strictly confidential. Any information leaving the hospital will have your name and address removed so that you cannot be recognised.

VII. Funding of the Study

This study is supported by funds through the University of Edinburgh

VIII. Results of the Study

The initial results of the research will be available about 2 years from the start of the study and will be published in peer reviewed medical journals. We do not expect our findings to alter the management of current patients; however it is hoped that they might reveal important correlations between blood clots and the level of microparticles, which may be of clinical relevance to patients in the future. We would thus like to be able to use the results we obtain from this study to compare with the results from other possible future studies. We will be happy to let you know our overall results at the completion of our study. If you would like to be informed in writing please let us know.

IX. New Information

If any new information becomes available during the course of this study, which might affect your willingness to continue taking part, your doctor will inform you immediately.

X. Contact for further information

If you require further information about this study, please contact Dr. Sharon Zahra at the New Royal Infirmary of Edinburgh on 0131 242 6096.

Thank you for taking the time to read this information sheet. Your participation in the study would be greatly appreciated and highly valued, as it would allow us to further our knowledge and help us treat future patients.

APPENDIX D

CONSENT FORM Study Number:

MICROPARTICLES AND THROMBIN GENERATION IN PATIENTS WITH GYNAECOLOGICAL MALIGNANCY

Patient Details (Addressograph Label)

Name:

Date of Birth:

CHI Number:

Please initial box

1. I confirm that I have read and understood the information sheet regarding the above-mentioned study, and have had the opportunity to ask questions. ☐
2. I understand that my participation is entirely voluntary and I am free to withdraw at any time without giving a reason, without my medical and legal rights being affected. ☐
3. I understand that the investigators of this study will inspect my medical records. ☐
4. I agree to take part in this study. ☐
5. I give permission for images and other information obtained in the course of this study to be used in any future publications. ☐
6. I agree to the notification of my GP regarding my participation in this study. ☐
7. I would like to be informed in writing of the overall findings of this study at its completion. ☐

_____	_____	_____
Name of patient	Date	Signature
_____	_____	_____
Name of Researcher	Date	Signature

Copy for patient, copy for researcher, copy to be kept with hospital notes

APPENDIX E

CONSENT FORM Study Number:

MICROPARTICLES AND THROMBIN GENERATION IN PATIENTS WITH BENIGN GYNAECOLOGICAL CONDITIONS

Patient Details (Addressograph Label)

Name:

Date of Birth:

CHI Number:

Please initial box

1. I confirm that I have read and understood the information sheet regarding the above-mentioned study, and have had the opportunity to ask questions. ☐
2. I understand that my participation is entirely voluntary and I am free to withdraw at any time without giving a reason, without my medical and legal rights being affected. ☐
3. I understand that the investigators of this study will inspect my medical records. ☐
4. I agree to take part in this study. ☐
5. I give permission for images and other information obtained in the course of this study to be used in any future publications. ☐
6. I agree to the notification of my GP regarding my participation in this study. ☐
7. I would like to be informed in writing of the overall findings of this study at its completion. ☐

_____	_____	_____
Name of patient	Date	Signature
_____	_____	_____
Name of Researcher	Date	Signature

Copy for patient, copy for researcher, copy to be kept with hospital notes

APPENDIX F: Publications arising from this thesis

1. **Levels of circulating microparticles in patients with gynaecological malignancy – a case control study**
   Zahra S, Anderson JAM, Stirling D, Ludlam CA
   Abstract and Poster
   British Society for Haematology, 50th Annual Scientific Meeting, 2010

2. **Circulating microparticles in patients with gynaecological malignancy**
   Zahra S, Anderson JAM, Stirling D, Ludlam CA
   Abstract and Poster
   15th Congress of the European Hematology Association, 2010

3. **Microparticles malignancy and thrombosis - review**
   Zahra S, Anderson JAM, Stirling D, Ludlam CA
   British Journal of Haematology 2011 Mar;152(6):688-700

4. **Plasma microparticles are not elevated in fresh plasma from patients with gynaecological malignancy – an observational study**
   Zahra S, Anderson JAM, Stirling D, Ludlam CA
   Gynecologic Oncology 2011 Oct; 123(1):152-6

5. **Study of the incidence of thrombotic events in patients with invasive gynaecological malignancies in the South East of Scotland**
   Zahra S, Zahra MA, Stirling D, Ludlam CA, Anderson JAM
   Abstract and Poster accepted at the 17th International Meeting of the European Society of Gynaecological Oncology, Milan, September 2011

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